CONTENTS

No. 1 MAY, 1949

CLARA MAE TAYLOR, ORREA FLORENCE PYE, ANNE BARMAN CALDWELL AND EDNA RUTH SOSTMAN. The energy expenditure of boys and girls 9 to 11 years of age (1) sitting listening to the radio (phonograph), (2) sitting singing, and (3) standing singing. One figure 1

MARJORIE M. NELSON AND HERBERT M. EVANS. Pteroylglutamic acid and reproduction in the rat 11

A. Z. HODSON. The microbiological assay of "folic acid" as applied to milk 25

J. W. BRATZLER, J. R. BARNES AND R. W. SWIFT. The effects of thiouracil on metabolism 41

HENRY CLARK SCHOR AND HARMON L. SWAIN. Simultaneous surveys of food consumption in various camps of the United States Army. Four figures 51

H. L. SWAIN, F. M. TOTH, F. C. CONSOLAZIO, W. H. FITZPATRICK, D. I. ALLEN AND C. J. KOEHN. Food consumption of soldiers in a subarctic climate (Fort Churchill, Manitoba, Canada, 1947–1948). One figure 63

J. WALTER WILSON, ELIZABETH H. LEDUC AND DIANE H. WINSTON. The production of biotin deficiency in the mouse. Two figures 73

SEDGWICK E. SMITH, M. A. WILLIAMS, A. C. BAUER AND L. A. MAYNARD. The wrist stiffness syndrome in guinea pigs 87

RICHARD J. BLANDAU, HANS KAUNITZ AND CHARLES A. SLANETZ. Ovulation, fertilization, and transport of ova in old, vitamin E deficient rats 97

No. 2 JUNE, 1949

J. R. Couch, V. H. Barki, M. L. Sunde, W. W. Cravens and C. A. Elvehjem. The utilization of lactose by the mature fowl 105

A. W. Halverson, Marie Zepplin and E. B. Hart. Relation of iodine to the goitrogenic properties of soybeans. Four figures 115

Gregory Shwartzman and Lotte Strauss. Vitamin B_6 deficiency in the Syrian hamster. Ten figures 131

Robert H. Silber and Curt C. Porter. Urinary excretion of amino acids and peptides by dogs fed protein hydrolysates or amino acids 155

Charles E. Calverley and Cornelia Kennedy. The effect of fat on calcium and phosphorus metabolism in normal growing rats under a normal dietary regime 165

G. B. Ramasarma, L. M. Henderson and C. A. Elvehjem. Purified amino acids as a source of nitrogen for the growing rat 177

A. L. Neumann, J. L. Krider, Marian F. James and B. Connor Johnson. The choline requirement of the baby pig. Thirty figures 195

Anthony A. Albanese, Selma E. Snyderman, Marilyn Lein, Emilie Smetak and Betty Vestal. The biological value of corn and wheat proteins in the male infant, with a note on the utilization of D-tryptophan 215

Marcella Garber, Mona M. Marquette and Helen T. Parsons. The availability of vitamins from yeasts. V. Differences in the influence of live yeast on the absorption of pure thiamine hydrochloride, pure riboflavin and nitrogen by human subjects, and the effect of distribution of the vitamin doses 225

Nevin S. Scrimshaw, Warren E. Porter and Mary W. Scrimshaw. The distribution of thiamine on the embryonated hen egg. I. The content of the whole embryo. One figure 237

Raymond Reiser and P. B. Pearson. The influence of high levels of fat with suboptimum levels of riboflavin on the growth of chicks. Three figures 247

P. B. Pearson and Frances Panzer. Effect of fat in the diet of rats on their growth and their excretion of amino acids 257

Nevin S. Scrimshaw, Warren E. Porter and Mary W. Scrimshaw. The distribution of thiamine in the embryonated hen egg. II. The content of embryonic tissues. One figure 267

No. 3 JULY, 1949

JAMES H. SHAW. Carious lesions in cotton rat molars. III. Effect of the particle size and the consistency of the purified ration on the incidence and type of carious lesions. Six figures ... 275

GUILLERMO H. MASSIEU, JESÚS GUZMAN, RENÉ O. CRAVIOTO AND JOSÉ CALVO. Determination of some essential amino acids in several uncooked and cooked Mexican foodstuffs ... 293

ANTHONY M. AMBROSE AND FLOYD DEEDS. The value of rutin and quercetin in scurvy. One figure ... 305

BACON F. CHOW, CARL ALPER AND SHIRLEY DEBIASE. The effect of retention of nitrogen in casein or lactalbumin hydrolysates on the regeneration of plasma proteins of protein-depleted dogs ... 319

WILLIAM S. HOFFMAN AND GORDON C. MCNEIL. The enhancement of the nutritive value of wheat gluten by supplementation with lysine, as determined from nitrogen balance indices in human subjects. One figure ... 331

CARYL B. HEIMER, HERMAN L. MASLOW AND ALBERT E. SOBEL. Influence of thyroid on utilization of vitamin A ... 345

ESBEN KIRK AND MARGARET CHIEFFI. Vitamin studies in middle-aged and old individuals. III. Thiamine and pyruvic acid blood concentrations. Three figures ... 353

HARRY J. DEUEL, JR., SAMUEL M. GREENBERG, EVELYN E. STRAUB, TOMOKO FUKUI, CHESTER M. GOODING AND CARL F. BROWN. Studies on the comparative nutritive value of fats. XI. On the possible growth-promoting activity of Δ^{13}-octadecenoic acid ... 361

HARRY J. DEUEL, JR., RICHARD M. JOHNSON, CLARENCE E. CALBERT, JANE GARDNER AND BIOLETTA THOMAS. Studies on the comparative nutritive value of fats. XII. The digestibility of rapeseed and cottonseed oils in human subjects ... 369

RALPH HOAGLAND, O. G. HANKINS, N. R. ELLIS AND G. G. SNIDER. Nutritive properties of protein in different cuts of beef ... 381

THE AMERICAN INSTITUTE OF NUTRITION. Proceedings of the Thirteenth Annual Meeting, Detroit, Michigan, April 18–22, 1949 ... 395

No. 4 AUGUST, 1949

LELAND C. CLARK, HELEN RYER AND JOHN R. MURLIN. Relative to egg biological values and replacement values of some cereal proteins in human subjects 405

BEULAH D. WESTERMAN, DOROTHY R. LINN, FRANCES TEMPLETON AND RUTH I. WELLS. Improving the nutritive value of flour. III. The use of enriched and non-enriched flour in diets similar to those consumed by certain low-income groups in South Carolina. Four figures 421

JIMMIE NELL HARRIS AND FLORENCE I. SCOULAR. Riboflavin metabolism of young women on self-selected diets 435

JUAN SALCEDO, JR., ALFONSO PEDROCHE, ELPIDIO C. PANGANIBAN AND JOSE F. DE LEON. Artificial enrichment of white rice as a solution to endemic beriberi. I. Preliminary report of field trials 443

BERTHA S. BURKE, STUART S. STEVENSON, JANE WORCESTER AND HAROLD C. STUART. Nutrition studies during pregnancy. V. Relation to maternal nutrition to condition of infant at birth: Study of siblings. Five figures 453

S. B. TOVE, A. E. SCHAEFER AND C. A. ELVEHJEM. Folic acid studies in the mink and fox. Two figures 469

FRANCES A. JOHNSTON, RUTH FRENCHMAN AND EUPHEMIA DEADE BOROUGHS. The iron metabolism of young women on two levels of intake 479

HANS KRINGSTAD AND SIGMUND FOLKVORD. The nutritive value of cod roe and cod liver. One figure 489

C. E. LATSCHAR, G. H. WISE, D. B. PARRISH AND J. S. HUGHES. Concentrations of various constituents in blood of dairy cows during stages of terminal gestation and initial lactation. I. Effect of prepartal diet on serum tocopherols 503

JOSÉ A. GOYCO AND CONRADO F. ASENJO. Net protein and growth-promoting values of three different types of yeast prepared under identical conditions. One figure 517

A. R. KEMMERER AND R. ACOSTA. The essential amino acid content of several vegetables 527

H. F. KRAUSE. A paradoxical relationship between serum level and liver content of vitamin A. One figure 535

ERRATA

The Journal of Nutrition, vol. 38, no. 1, May, 1949.

A. Z. HODSON

Page 27, table 1, under "Fresh Milk 3" and under "Evaporated Milk 3": the word *and* should be inserted after "acids" so the two passages will read "Proteins precipitated with hydrochloric and metaphosphoric acids and at pH 7.0."

Pages 32 and 33, tables 4 and 5: the numerical values should read $\mu g/l$ instead of $\mu g/gm$.

THE ENERGY EXPENDITURE OF BOYS AND GIRLS 9 TO 11 YEARS OF AGE (1) SITTING LISTENING TO THE RADIO (PHONOGRAPH), (2) SITTING SINGING, AND (3) STANDING SINGING

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ONE FIGURE

(Received for publication January 7, 1949)

Among the many activities in which 9- to 11-year-old boys and girls engage, that of sitting listening to the radio has been found to be one of the favorite indoor pastimes. Children also sing a great deal both at home and at school. In the interest of increasing our knowledge of the energy requirements of children, and in view of the fact that the energy expenditure of children engaged in these activities had not been previously investigated, such studies were undertaken.

EXPERIMENTAL

Subjects

The children came from backgrounds similar to those of the children reported on in studies by Taylor, Pye and Caldwell ('48). Although a number of the same children participated in the study of each of the three activities, it was necessary to work with new children from time to time since some grew out of the age range and a good representation of children within the age range was desired. In all, 22 children

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TABLE 1

Average weight, height, deviation from predicted weight, and basal metabolism of children studied (figures in parentheses show range)

SUBJECTS	BODY WEIGHT	BODY HEIGHT	DEVIATION FROM PREDICTED WEIGHT [1]	BASAL METABOLISM IN CALORIES PER			
				24 hr.	kg/hr.	m^2/hr.	cm of ht./24 hr.
	kg	*cm*	%				
Boys,[2] Ages 9 yr. 4 mo. to 11 yr. 5 mo.							
Group I							
12 boys serving as subjects for listening to radio	37.6 (30.8–46.2)	143.9 (136.5–153.3)	+ 3.0 (— 7.9–+ 14.5)	1287 (1186–1543)	1.44 (1.21–1.69)	43.7 (39.2–48.8)	8.9 (8.3–10.3)
Group II							
6 boys serving as subjects for sitting singing	36.8 (31.6–44.1)	144.1 (136.5–150.6)	+ 1.6 (— 7.9–+ 12.0)	1306 (1186–1426)	1.45 (1.35–1.63)	44.6 (42.8–48.8)	9.1 (8.5–9.7)
Group III							
8 boys serving as subjects for standing singing	37.0 (31.6–44.1)	144.1 (136.5–151.0)	+ 0.9 (— 7.9–+ 12.0)	1285 (1186–1426)	1.49 (1.35–1.63)	43.8 (40.5–48.8)	8.9 (8.3–9.7)
Girls,[2] Ages 8 yr. 9 mo. to 10 yr. 11 mo.							
Group I							
10 girls serving as subjects for listening to radio	33.4 (28.1–41.1)	137.6 (126.8–149.6)	+ 2.1 (— 8.3–+ 14.3)	1191 (1114–1331)	1.50 (1.34–1.67)	43.9 (41.4–48.3)	8.7 (8.1–9.4)
Group II							
9 girls serving as subjects for sitting and standing singing	35.5 (29.3–50.0)	140.6 (126.8–156.0)	+ 2.4 (— 8.3–+ 11.7)	1228 (1105–1531)	1.46 (1.28–1.67)	43.5 (41.4–48.3)	8.7 (8.1–9.8)

[1] Deviations from Baldwin-Wood standards.

[2] Many of the same boys — or girls — were subjects for each activity. In cases where they outgrew the age range or were unavailable before sufficient tests on a given activity were obtained, additional subjects were included.

(12 boys and 10 girls) served as experimental subjects for the studies of the energy expenditure of children sitting listening to the radio (phonograph), 15 children (6 boys and 9 girls) for sitting singing, and 17 children (8 boys and 9 girls) for standing singing. Health records reported by physicians were consulted to determine the general health status of the children before they were asked to serve as experimental subjects. The weight, height, and deviations from predicted weight for height and age (Baldwin-Wood standards) were determined. The average values are given in table 1. Although the children varied in national background, they were all born in this country.

Procedure

Basal metabolism determinations were made on all subjects under standard conditions at the beginning of the experimental period by means of the Collins-Benedict-Roth respiration apparatus. Not less than two determinations on each of two mornings were made on each child. More determinations were made when necessary in order to obtain an average of three of the lowest results agreeing within 5%. The activity studies in each case were completed within a period of less than 6 months following the basal metabolism determination. Whenever an experimental subject was continued for a longer period than this, another basal metabolism determination was made. The children included in the study had basal metabolisms which agreed within the commonly accepted normal range of $\pm$ 15% with two or more of the standards currently used for children.

The open-circuit respiration chamber described by Taylor, Lamb, Robertson and MacLeod ('48) was used for the activity studies. Two boys or two girls were studied at a time. The total carbon dioxide produced was divided by two to obtain the average carbon dioxide production per child and heat production computed as described in the paper just cited. The Carpenter modification of the Haldane gas analysis ap-

paratus was used for determining the carbon dioxide content of chamber air.

The activity studies were made after school. Since it was not possible to use a radio in the respiration chamber, programs for children similar to those on the radio had to be especially planned for each test. A phonograph was used and suitable records were selected from the Teachers College library collection for 9- to 11-year-old children. The phonograph was operated by an observer outside of the chamber and the stories or songs were broadcast into the chamber by means of an army two-way speaking system such as was used for communication in tanks and planes during the war (fig. 1). A field phone was placed in the chamber at such an elevation as to provide clear reception for the children in a comfortable sitting position. The same equipment was used for the study of the energy expenditure of children sitting listening to the recordings, and sitting and standing singing.

All tests were preceded by a 10-minute rest period. During the rest period the children sat quietly reading or playing with puzzles, games, or engaging in some other activity involving only finger muscles. Sometimes the rest period was followed by a period of sitting listening to the phonograph and at other times by one of the singing periods.

The storytelling period was planned for 20 minutes but the actual time varied from 18 to 23 minutes, depending upon the length of the story record and whether the voice of the storyteller necessitated an adjustment of the speed to insure clarity. The children sat and listened attentively, often spontaneously making gestures in an attempt to dramatize parts of the story. The records had to be carefully timed for the proper speed and the afternoon program rehearsed by the observers in advance so that the experimental period would approximate 20 minutes. Care was taken to avoid stories which were too young for the older children in the group in order to eliminate the possibility of muscle tension due to boredom.

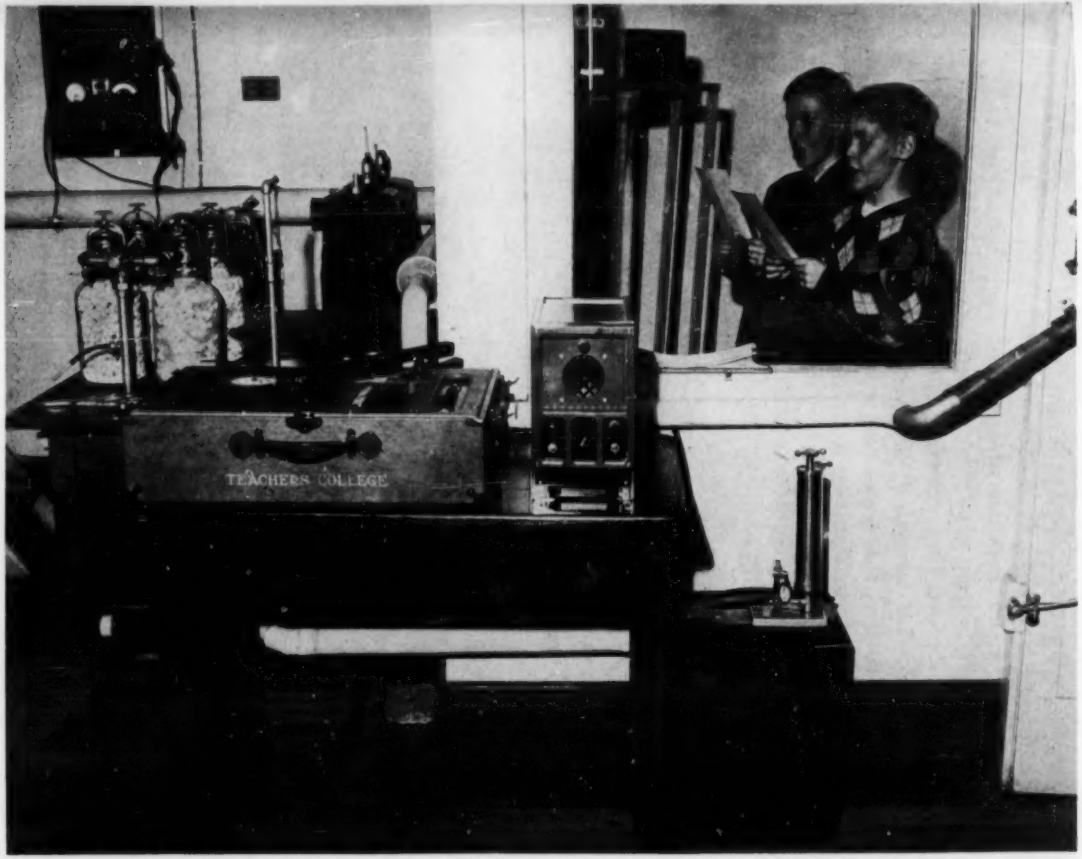

Fig. 1 Two young boys standing singing in the respiration chamber. The two-way system is shown on table in front of respiration chamber, along with phonograph. The field phone, mounted on standard, can be seen through chamber window.

The same care was necessary in the selection of the songs for the singing periods. The program for the singing periods also had to be carefully timed and rehearsed by the observers in advance for an experimental period approximating 10 minutes. The children were given typed copies of the words of the songs before they entered the respiration chamber, and the recorded voice was clear enough for the children to follow easily so that there was continuous singing during the experimental period. The two activities, (1) sitting singing and (2) standing singing, were never studied on the same afternoon because the children could not be depended upon to sing enthusiastically and continuously for longer than 10 minutes at a time.

RESULTS AND DISCUSSION

The average results of these studies expressed in terms of total Calories per hour per kilogram of body weight and per centimeter of height are given in table 2.

(1) Listening to the phonograph

The average energy expenditure of the boys when sitting listening to the phonograph was 2.07 ± 0.06 Cal. per kilogram of body weight per hour and 0.54 ± 0.01 Cal. per centimeter of height per hour, and of the girls 1.80 ± 0.04 Cal. per kilogram of body weight per hour and 0.45 ± 0.01 Cal. per centimeter of height per hour. It will be noted that the boys spent more energy than the girls when engaging in the same activity. This is in line with the results of other studies from this laboratory. The coefficients of variation are, respectively, 13.0% and 11.9% for the boys, and 10.5% and 12.8% for the girls. When the probable error is expressed as per cent of the mean it is small, being only 2.8% and 2.6% for the boys and 2.0% and 2.5% for the girls. It is the opinion of the authors, resulting from observations of children sitting listening to the radio under nonexperimental conditions, that the amount of activity in which the children engaged under the

TABLE 2

Average energy expenditure of children 9 to 11 years old, for (1) listening to the radio (phonograph), (2) sitting singing, and (3) standing singing (including the basal metabolism)

	LISTENING TO THE RADIO (PHONOGRAPH)		SITTING SINGING		STANDING SINGING	
	Boys (10 cases)	Girls (12 cases)	Boys (10 cases)	Girls (12 cases)	Boys (11 cases)	Girls (13 cases)
CO_2 (gm) produced per child per hr.						
Mean ± P.E.[1]	26.06±0.75	20.48±0.64	27.05±0.89	24.39±0.82	28.78±0.78	25.09±0.56
C.V.[2] (%)	13.6	16.1	15.4	17.2	13.3	12.0
P.E. as % mean	2.9	3.1	3.3	3.4	2.7	2.2
Cal. per kg per hr.						
Mean ± P.E.	2.07±0.06	1.80± 0.04	2.23±0.06	2.06±0.07	2.35±0.05	2.13±0.06
C.V. (%)	13.0	10.5	13.2	17.5	10.7	14.2
P.E. as % mean	2.8	2.0	2.8	3.4	2.2	2.7
Cal. per cm ht. per hr.						
Mean ± P.E.	0.54±0.01	0.45 ±0.01	0.57±0.02	0.52±0.02	0.60±0.01	0.54±0.01
C.V. (%)	11.9	12.8	14.0	15.9	11.6	11.2
P.E. as % mean	2.6	2.5	3.0	3.1	2.4	2.1

[1] Probable error.

[2] Coefficient of variation.

experimental conditions of this study closely approximated that of the nonexperimental children, even though some of the programs might well be judged less exciting than many radio programs for children.

(2) Sitting singing

The average energy expenditure of the boys for sitting singing was 2.23 ± 0.06 Cal. per kilogram of body weight per hour and 0.57 ± 0.02 Cal. per centimeter of height per hour, and of the girls 2.06 ± 0.07 Cal. per kilogram of body weight per hour and 0.52 ± 0.02 Cal. per centimeter of height per hour. The energy expenditure of the boys was slightly above that of the girls, and the energy expenditure for sitting singing of both boys and girls was above that of both for sitting listening to the radio, although the difference is very small in each case. Although the children were not always equally enthusiastic about singing, on the whole both boys and girls cooperated well. All precautions were taken to insure the interest of the children and to duplicate, as far as possible, actual situations.

Studies of the energy expenditure of boys of this age seated and engaged in quiet play have been reported by Taylor ('37), and of girls by Potgieter ('33) and Thompson ('40). Other studies of boys and girls of this age engaged in other activities where the children were seated have been reported by Sondén and Tigerstedt (1895), Olin ('16), and Bedale ('23). The findings of all of these investigators where the children were seated performing different tasks but with similar degrees of activity are of the same order of magnitude as those of this study and will be discussed in detail in a later paper from this laboratory.

(3) Standing singing

The average energy expenditure of the boys standing singing was 2.35 ± 0.05 Cal. per kilogram of body weight per hour and 0.60 ± 0.01 Cal. per centimeter of height per hour, and

of the girls 2.13 ± 0.06 Cal. per kilogram of body weight per hour and 0.54 ± 0.01 Cal. per centimeter of height per hour. The coefficients of variation are of the same order of magnitude as for the other activities, with a slight tendency to be lower. When the probable error of the mean is expressed as per cent of the mean it again is small, as shown in table 2. The energy expenditure for standing singing for both boys and girls is only slightly above that for sitting singing. The children stood quietly for the most part with occasional swaying and sang with varying degrees of enthusiasm. A few of the children responded to some of the songs having certain rhythms by tapping their feet while singing. Potgieter's studies ('33) of the energy expenditure of girls of this age range standing relaxed are in agreement with the results of this study.

SUMMARY

The energy expenditure of 22 children (12 boys and 10 girls), 9 to 11 years of age, was measured while they were sitting in a respiration chamber listening to the phonograph. The average energy expenditure of the boys was 2.07 ± 0.06 Cal. per kilogram of body weight per hour (coefficient of variation, 13.0%) and 0.54 ± 0.01 Cal. per centimeter of height per hour (coefficient of variation, 11.9%); and of the girls 1.80 ± 0.04 Cal. per kilogram of body weight per hour (coefficient of variation, 10.5%) and 0.45 ± 0.01 Cal. per centimeter of height per hour (coefficient of variation, 12.8%).

The energy expenditure of 15 children (6 boys and 9 girls) was measured while they were sitting singing. The average energy expenditure of the boys was 2.23 ± 0.06 Cal. per kilogram of body weight per hour (coefficient of variation, 13.2%) and 0.57 ± 0.02 Cal. per centimeter of height per hour (coefficient of variation, 14.0%); and of the girls 2.06 ± 0.07 Cal. per kilogram of body weight per hour (coefficient of variation, 17.5%) and 0.52 ± 0.02 Cal. per centimeter of height per hour (coefficient of variation, 15.9%).

The energy expenditure of 17 children (8 boys and 9 girls) was measured while they were standing singing. The average energy expenditure of the boys was 2.35 ± 0.05 Cal. per kilogram of body weight per hour (coefficient of variation, 10.7%) and 0.60 ± 0.01 Cal. per centimeter of height per hour (coefficient of variation, 11.6%); and of the girls 2.13 ± 0.06 Cal. per kilogram of body weight per hour (coefficient of variation, 14.2%) and 0.54 ± 0.01 Cal. per centimeter of height per hour (coefficient of variation, 11.2%).

LITERATURE CITED

BEDALE, E. M. 1923 Energy expenditure and food requirements of children at school. Proc. Roy. Soc., London, B, *94:* 368.

OLIN, H. 1916 Über die kohlenstoffabgabe bei knaben im alter von 10 bis 18 jahren. Skand. Arch. Physiol., *34:* 414.

POTGIETER, M. 1933 The energy cost of some forms of physical activity of 9-year-old girls. Dissertation, Columbia University, New York City.

SONDÉN, K., AND R. TIGERSTEDT 1895 Untersuchungen über die respiration und gesammtstoffwechsel des menschen. Skand. Arch. Physiol., *6:* 1.

TAYLOR, C. M. 1937 The energy metabolism and mechanical efficiency of young boys. Dissertation, Columbia University, New York City.

TAYLOR, C. M., M. W. LAMB, M. E. ROBERTSON AND G. MACLEOD 1948 The energy expenditure of 7- to 14-year-old boys for quiet play and cycling. J. Nutrition, *35:* 511.

TAYLOR, C. M., O. F. PYE AND A. B. CALDWELL 1948 The energy expenditure of 9- to 11-year-old boys and girls (1) standing drawing and (2) dressing and undressing. Ibid., *36:* 123.

THOMPSON, E. 1940 A study of the energy expenditure and mechanical efficiency of young girls and adult women. Dissertation, Columbia University, New York City.

PTEROYLGLUTAMIC ACID AND REPRODUCTION IN THE RAT [1]

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(Received for publication December 18, 1948)

Nelson and Evans ('47) have shown that on the day of breeding a young female rat may be fed a purified diet containing succinylsulfathiazole (SST) and supplemented with all the known vitamins with the exception of pteroylglutamic acid (PGA) and proceed normally with this gestation. When this PGA-deficient diet was fed one to three months prior to breeding, marked upsets in reproduction occurred, i.e., resorption, decreased number of young per litter, and increased percentage of young born dead. Rats three to 4 months of age were used in this study.

The present communication reports (1) the reproductive performance of rats in which the deficiency was instituted at an earlier age (one to two months), (2) the accentuating effects of a chemical antagonist of pteroylglutamic acid, and (3) the curative effects of synthetic PGA. Consideration of

[1] Aided by grants from the Roche Anniversary Foundation, the Board of Research and the Department of Agriculture of the University of California, and the Rockefeller Foundation, New York City. We are indebted to Dr. T. H. Jukes, Lederle Laboratories, Inc., Pearl River, New York, for generous supplies of synthetic pteroylglutamic acid and of the chemical antagonist for pteroylglutamic acid; to Dr. W. A. Feirer of Sharp and Dohme, Inc., Glenolden, Pennsylvania, for succinylsulfathiazole; to Dr. E. L. Sevringhaus of Hoffmann-LaRoche, Inc., Nutley, New Jersey, for crystalline *d*-biotin; and to Dr. Randolph Major of Merck and Company, Inc., Rahway, New Jersey, for crystalline B vitamins, alpha-tocopherol and 2-methyl-1, 4-naphthoquinone.

the possible role of diminished food intake was obviated by the institution of paired feeding controls.

EXPERIMENTAL

Normal female rats (Long-Evans strain) from one to three months of age were placed on the PGA-deficient diet and bred with normal males after one, two or three months of the deficiency. Additional groups of animals, three to 4 months of age, were bred with normal males and placed on the deficient or supplemented diet the day of mating. Vaginal smears were examined daily during gestation for the presence of erythrocytes, the sign that implantation has occurred; all rats were weighed at regular intervals. The animals were kept on screens until the day before littering was expected to occur. All rats were autopsied a few hours after parturition had taken place, except when weight loss indicated resorption had begun autopsies were performed as early as the 20th day of gestation. The uterus was carefully examined for the presence of implantation or resorption sites.

The PGA-deficient diet was the same as that used previously by the present authors (Nelson and Evans, '47). It consisted of 24% alcohol-extracted casein, 63% sucrose, 8% hydrogenated cottonseed oil,[2] 4% salts No. 4, and 1% succinylsulfathiazole (SST). Crystalline vitamins per kilogram of diet were: 300 μg *d*-biotin, 5 mg 2-methyl-1, 4 naphthoquinone, 5 mg thiamine HCl, 5 mg pyridoxine HCl, 10 mg riboflavin, 10 mg *p*-aminobenzoic acid, 20 mg nicotinic acid, 50 mg *d*-calcium pantothenate, 400 mg inositol and 1.0 gm choline chloride. Control rats received the identical diet supplemented with 5.5 mg synthetic PGA per kilogram of diet. Each rat received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vitamin A, 115 Chick Units vitamin D, 6 mg synthetic alpha-tocopherol, and 650 mg corn oil.[3]

The chemical antagonist of PGA used was that described by Franklin et al. ('47a), who reported that this "crude" syn-

[2] Crisco or Primex.
[3] Mazola.

thetic preparation [4] nullified otherwise adequate amounts of pteroylglutamic acid in the case of *Streptococcus faecalis R, Lactobacillus casei,* and the rat. Franklin, Stokstad and Jukes ('47b) described the acceleration of PGA deficiency by this preparation in mice and in chicks. Additional observations of its effects on the chick have been made by Hertz ('48c) and on the pig by Welch et al. ('47).

RESULTS

Table 1 summarizes the reproductive preformances of rats of different ages after varying periods of PGA-deficiency. The data for additional rats, 4 months old, placed on the purified SST diets the day of breeding are given to show again that this procedure does not impair the reproductive mechanism. It will be noted that there is little interference with implantation, as only one deficient group (after two months of prior deficiency) shows a failure of implantation greater than 10%. The average failure of implantation for all groups with deficiency prior to breeding (85 litters) is 7%, the identical value for all groups placed on the experimental diets the day of breeding (42 litters).

Although the variability among groups is marked, the data show that one month of prior deficiency, regardless of the age of the rats, interferes slightly with reproductive performance; two months markedly; and three months less markedly. Eleven per cent (range 0–25) of the rats resorbed after one month on the deficient diet, 44% (range 29–56) after two months, and 22% (range 13–33) after three months of prior deficiency. This decreased incidence of resorption after three months suggests some adjustment to the diet made during this prolonged period. There is little correlation with age and incidence of resorptions. The youngest rats (30–35 days old at the beginning of the experimental period) had the highest percentage of resorptions after one and two months

[4] Lot no. 125 was used in these studies without further purification.

TABLE 1

Effect of pteroylglutamic acid (PGA) deficiency on reproduction

TIME DIETS STARTED	RATS BRED	AGE WHEN BRED	BODY WT. WHEN BRED	FAILED IMPLANTATION	RESORPTIONS OF IMPLANTATIONS	LITTERS OF IMPLANTATIONS	TOTAL YOUNG	YOUNG BORN DEAD	AV. YOUNG PER LITTER	AV. WT. OF YOUNG
	no.	*days*	*gm*	%	%	%	no.	%	no.	*gm*
				PGA-supplemented rats						
Day of	12	129	217	17	0	100	81	0	8.1	5.5
breeding	10	130	219	0	0	100	87	0	8.7	5.9
Average	22	129	218	9	0	100	168	0	8.4	5.7
				PGA-deficient rats						
Day of	12	128	218	8	0	100	101	4	9.2	5.2
breeding	12	130	221	0	0	100	112	0	9.3	5.5
Average	24	129	220	4	0	100	213	2	9.3	5.4
34 days	9	65	178	10	25	75	35	11	5.0	4.3
before	10	100	219	10	0	100	82	4	9.1	4.7
breeding	10	127	230	0	10	90	64	9	7.1	4.7
Average	29	95	210	7	11	89	181	8	7.3	4.6
63 days	8	94	228	0	56	44	18	0	4.5	4.7
before	10	130	224	30	29	71	36	6	7.1	5.5
breeding	9	156	245	0	44	56	29	3	5.8	5.8
Average	27	128	232	11	44	56	83	3	6.0	5.4
94 days	8	124	222	0	13	87	45	4	6.4	5.4
before	10	162	247	0	20	80	46	4	5.8	5.3
breeding	10	184	260	10	33	67	32	6	5.3	5.5
Average	28	159	245	3	22	78	123	4	5.8	5.4

of deficiency but the lowest after three months. Resorptions, according to the beginning age of the animals, averaged 31% for those starting at one month, 16% for those two months old, and 29% for those three months old. It is possible that the intermediate group of rats (two months of age) may have had greater vitamin stores than the other groups for some reason.

The percentage of young born dead is consistently higher than normal (i.e., 8%, 3% and 4%) after one, two, or three months of prior deficiency. The average number of young per litter is slightly decreased after one month of deficiency (7.3 young) and significantly decreased after two or three months of prior deficiency (6.0 and 5.8 young, respectively). The average weight of the young at birth is significantly decreased only for the group with one month of prior deficiency.

Effect of undernutrition and PGA supplementation

In order to evaluate the role of undernutrition and the curative effects of PGA supplementation on these reproductive upsets, paired feeding studies were carried out. The rats were placed on the PGA-deficient diet at weaning (21 days) and bred with normal males after 60 days of the deficiency. (In the preceding study two months of prior deficiency had resulted in the highest incidence of resorptions.) One-third of the animals were continued on the PGA-deficient diet and their food intake measured daily; one-third received the PGA-supplemented diet in the same amount as was consumed by the corresponding deficient rats on the same day of gestation; and the remaining one-third received the PGA-supplemented diet ad libitum. The rats were carefully paired [5] with respect to age and body weight on the day of breeding

[5] The PGA-deficient group averaged 85 days of age and 199 gm in body weight the day of breeding after 63 days of deficiency with an average weight gain of 153 gm. The corresponding averages for the control groups were as follows: pair-fed controls — 85 days, 196 gm; 64 days, 150 gm; ad libitum controls — 86 days, 196 gm; 65 days, 151 gm; ad libitum group receiving the PGA-supplemented diet throughout the entire experimental period — 85 days, 199 gm; 64 days, 153 gm.

and with regard to length of deficiency period and weight gain during this time. A fourth group was selected from additional animals placed on the PGA-supplemented diet at weaning and likewise bred after 60 days on the experimental diet.

The experimental data are summarized in table 2. In the deficient group two rats (20%) resorbed while the remainder (80%) littered. This incidence of resorptions is considerably lower than that previously found after two months of deficiency and emphasizes the variability to be expected. The average weight of the deficient young at birth was significantly lower than normal but the number of young per litter and the percentage of young born dead were not adversely affected.

In the pair-fed group, as in all other PGA-supplemented groups, 100% of the rats littered, showing that limitation of food intake is not the cause of resorptions in the deficient group. The birth weight of the young from pair-fed mothers was significantly higher than that of deficient young but did not attain the normal birth weights of the ad libitum groups. The restriction in food intake varied from 52 to 92% (average 76%) of the food eaten by the rats given the PGA-supplemented diet ad libitum. The increased efficiency of food utilization by rats receiving the synthetic vitamins is only slight (no significant difference), as can be seen by comparing the weight changes during the 20-day gestation period of the deficient and pair-fed groups, i.e., 45 gm and 55 gm, respectively. This is to be expected, inasmuch as the deficiency produced by the experimental procedure was only slight and resulted in a low incidence of resorptions.

The high percentage of young born dead in the two control groups subjected to the deficiency for two months prior to breeding should be noted. Although these high values (18% for the pair-fed group and 13% for the ad libitum group) are due principally or entirely to one dead litter in each group, they suggest that the addition of the synthetic vitamin, at the

TABLE 2

Effects of food restriction and of PGA supplementation on reproduction with 64 days of PGA deficiency [1] prior to breeding

EXPTL. GROUP	RATS BRED	BODY WT. AT MATING	WT. CHANGE DURING GESTATION [2]	AV. DAILY FOOD INTAKE [3]	FAILED IMPLANTATION	RESORPTIONS OF IMPLANTATIONS	LITTERS OF IMPLANTATIONS	TOTAL YOUNG	YOUNG BORN DEAD	AV. YOUNG PER LITTER	AV. WT. YOUNG
	no.	*gm*	*gm*	*gm*	%	%	%	*no.*	%	*no.*	*gm*
PGA-deficient	10	199	+ 45 ± 7.2 [4]	11.5 ± 0.6	0	20	80	66	2	8.3 ± 0.9	3.9
PGA-supplemented pair-fed controls	10	196	+ 55 ± 5.5	11.5 ± 0.6	0	0	100	71	18	7.1 ± 0.5	4.7
PGA-supplemented ad libitum controls	10	196	+ 95 ± 6.9	15.2 ± 0.6	0	0	100	80	13	8.0 ± 0.7	5.7
PGA-supplemented ad libitum controls [1]	10	199	+ 89 ± 2.7	14.5 ± 0.4	0	0	100	92	0	9.2 ± 0.6	5.5

[1] The first three groups received the PGA-deficient diet 64 days prior to breeding and these two supplemented groups show the curative effects of the vitamin; the 4th group received the PGA-supplemented diet throughout the entire experimental period and demonstrates the prophylactic effects of the vitamin.

[2] Data determined during the 20 days following mating.

[3] Data determined during 21–22 days following mating.

[4] Standard deviation of the mean.

level used, only during gestation was not sufficient to counteract all the effects of the prior deficiency period. There was complete normality of the young from the control group receiving the vitamin prior to breeding.

Effect of the antagonist

To accentuate the deficiency during reproduction, 5 gm of the "crude" antagonist per kilogram (i.e., 0.5%)[6] were added to the PGA-deficient diet. Normal females, three months of age, were placed on the deficient diet 10 to 15 days prior to breeding or on the day of breeding. Control rats received the identical diet supplemented with 50.5 mg synthetic PGA per kilogram diet, i.e., almost 10 times the usual level of 5.5 mg per kilogram diet.

Table 3 shows that the addition of 0.5% of the antagonist to the PGA-deficient diet results in 100% resorptions either with or without a prior deficiency period. PGA supplementation during gestation was not sufficient to counteract completely the effects of a prior deficiency period, as shown by the occurrence of resorption in this group; in addition, the number of young per litter was decreased slightly. When there was no prior deficiency period, PGA supplementation resulted in 100% litters with a normal number of young per litter. The marked differences in body weight change during gestation for the PGA-deficient and supplemented groups should be noted, as well as the fact that the deficient rats resorbed despite the gain in weight during the gestation period.

[6] Preliminary studies had shown that the addition of 1 gm of the antagonist per kilogram diet had little effect on reproduction. The addition of 10 gm antagonist per kilogram diet resulted in an acute PGA deficiency and 100% of the rats placed upon such a diet the day of breeding resorbed instead of littering. This acute deficiency in adult rats was characterized by an early loss of the estrous cycle in 15–30 days, a marked weight loss after 20 days, and death in 30–50 days. A marked anemia and leukopenia (especially granulocytopenia), severe oral lesions and deposition of pigment were noted in agreement with the observations of Franklin et al. ('47a) on weanling rats receiving similar levels of the antagonist.

TABLE 3

Effect of the antagonist on PGA deficiency during reproduction

TIME DIETS STARTED	RATS	BODY WT. WHEN BRED	WT. CHANGE DURING GESTATION [1]	FAILED IMPLANTATION	RESORPTIONS OF IMPLANTATIONS	LITTERS OF IMPLANTATIONS	TOTAL YOUNG	YOUNG BORN DEAD	AV. YOUNG PER LITTER	AV. WT. YOUNG
	no.	*gm*	*gm*	*%*	*%*	*%*	*no.*	*%*	*no.*	*gm*
				PGA-deficient rats						
13 days before breeding	15	248	— 12	13	100	0				
Day of breeding	13	229	+ 24	0	100	0				
	13	208	+ 20	0	100	0				
	11	225	+ 18	0	100	0				
	10	223	+ 23	0	100	0				
				PGA-supplemented rats						
Day of breeding (13 days prior deficiency)	15	247	+ 96	0	7	93	104	1	7.4	5.5
Day of breeding	11	233	+ 119	0	0	100	118	5	10.7	5.5
	10	223	+ 114	0	0	100	111	2	10.4	5.7

[1] Data determined during the 20 days following mating.

TABLE 4

Effect of food restriction and PGA supplementation on reproduction in the presence of the antagonist

EXPTL. GROUP	RATS BRED	BODY WT. AT MATING	WT. CHANGE DURING GESTATION [1]	AV. DAILY FOOD INTAKE [2]	FAILED IMPLANTATION	RESORPTIONS OF IMPLANTATIONS	LITTERS OF IMPLANTATIONS	TOTAL YOUNG	YOUNG BORN DEAD	AV. YOUNG PER LITTER	AV. WT. YOUNG
	no.	gm	gm	gm	%	%	%	no.	%	no.	gm
PGA-deficient	10	223	+ 23 ± 6.9 [3]	14.0 ± 0.5	0	100	0				
PGA-supplemented pair-fed controls	10	224	+ 79 ± 8.9	14.0 ± 0.5	0	0	100	105	1	9.0 ± 0.7	5.4
PGA-supplemented ad libitum controls	10	223	+ 114 ± 6.3	17.9 ± 0.6	0	0	100	111	2	10.4 ± 0.4	5.8

[1] Data determined during the 20 days following mating.
[2] Data determined during the 22 days following mating.
[3] Standard deviation of the mean.

Effect of food restriction on reproduction in the presence of the antagonist

To eliminate the factor of undernutrition as a cause of this marked reproductive failure in the presence of the antagonist, pair-fed controls were added in line with the above experimental procedure. The rats were carefully paired in regard to age and body weight on the day of breeding, as follows: PGA-deficient group, 101 days of age and 223 gm in body weight; pair-fed group, 106 days and 224 gm; ad libitum group, 105 days and 223 gm.

Table 4 presents the experimental data in detail. One hundred per cent of the PGA-deficient group resorbed, whereas the pair-fed group littered 100%. The restriction in food intake varied from 69 to 98% (average 79%) of the food eaten by rats given the PGA-supplemented diet ad libitum. The marked increase in efficiency of food utilization by the rats receiving the vitamin can be seen by comparing the weight changes during the 20-day gestation period, i.e., 23 gm for the deficient group and 79 gm for the pair-fed animals. Neither the deficiency nor the restriction in food intake during the gestation period interfered with implantation, as is shown by the normal number of implantation sites per rat for all groups. The food restriction did not affect the number of young per litter nor their average weight at birth. However, the group receiving the PGA-supplemented diet ad libitum gained much more during gestation than did the pair-fed group.

DISCUSSION

The data presented in this study demonstrate that pteroylglutamic acid is essential for reproduction in the rat when SST is incorporated in the diet. The deficiency produced in adult rats by the addition of SST to a purified diet lacking in the vitamin is only slight, as shown by the low incidence of resorptions (26% for 85 rats) and the length of deficiency prior to gestation required to produce the reproductive upsets.

The further addition of 0.5% of the chemical antagonist produces a very acute deficiency and reproductive failure occurs during the three-week gestation period. The normality of reproductive performance in PGA-supplemented controls restricted in food intake eliminates the factor of undernutrition and of other dietray deficiencies as a cause of these failures in reproduction. The lack of correlation between food intake and reproductive performance is further shown by the marked difference in reproductive success in the presence and absence of the antagonist with very little difference in food intake. In the presence of the antagonist 100% of the rats resorbed with an average 79% restriction, whereas in the absence of the antagonist 20% of the rats resorbed with an average 76% restriction in food intake. The limitation of food intake for either group was not as great as that previously reported for pantothenic acid deficiency during reproduction, i.e., 69% (Nelson and Evans, '46).

In regard to the specific function of pteroylglutamic acid in the reproductive mechanism of the rat, not much can be said at this time. Little interference with implantation was noted in the deficient groups except after prolonged periods of the deficiency prior to breeding. This is in marked contrast with the noticeable interference with implantation in the case of pantothenic acid deficiency (Nelson and Evans, '46) and that produced in pyridoxine deficiency with the aid of the potent vitamin antagonist, desoxypyridoxine (Nelson and Evans, '48). In pyridoxine deficiency, moreover, an early appearance of the erythrocyte sign was frequently observed. In this study the erythrocyte sign occurred normally on the 13th or 14th day of gestation, even for deficient animals receiving 0.5% of the antagonist. Furthermore, such animals gained weight up to the 13th day or even later and then lost slightly by the 20th day, so that a net gain in body weight resulted during the gestation period despite the simultaneous occurrence of resorption, a process practically completed by the 20th day as determined by examination of the uterus at autopsy. The findings of Hertz and Sebrell ('44) and

Hertz ('45, '48a, '48b) that the characteristic tissue-growth responses to estrogen in the genital tract of both the female monkey and the chick require an adequate intake of pteroylglutamic acid are suggestive, as well as the recent report (Hertz, 48c) that the addition of the chemical antagonist (the same preparation used in this study) to a stock diet interferes with this response in the chick.

SUMMARY

Reproduction has been studied in adult rats placed on purified SST diets deficient in pteroylglutamic acid prior to the gestation period. In confirmation of previous studies, the impairment in reproductive performance was greatest after two months of deficiency prior to breeding; less marked effects were noted after three months of prior deficiency. There was little correlation between reproductive impairment and the age of the animals when the deficiency was started.

Pair-fed animals supplemented with the vitamin demonstrated that undernutrition was not the cause of resorptions under the prevailing experimental conditions. The level of synthetic pteroylglutamic acid given during gestation was not sufficient to counteract completely the effects of prior deficiency on the young, whereas young from mothers receiving the vitamin throughout the entire experimental period were in all respects normal.

The addition of 0.5% of a chemical antagonist markedly accentuated the vitamin deficiency and resulted in 100% resorptions for adult rats even without any prior deficiency period. The complete normality of pair-fed controls supplemented with high levels of the vitamin eliminated the factors of undernutrition and of other specific dietary deficiencies as causes for these reproductive failures.

ACKNOWLEDGMENT

The authors wish to express their appreciation for the able technical assistance of Miss Dorothy Cozens and Mr. Jack Rushmere in carrying out this study.

LITERATURE CITED

FRANKLIN, A. L., E. L. R. STOKSTAD, M. BELT AND T. H. JUKES 1947a Biochemical experiments with a synthetic preparation having an action antagonistic to that of pteroylglutamic acid. J. Biol. Chem., *169:* 427.

FRANKLIN, A. L., E. L.R. STOKSTAD AND T. H. JUKES 1947b Acceleration of pteroylglutamic acid deficiency in mice and chicks by a chemical antagonist. Proc. Soc. Exp. Biol. Med., *65:* 368.

HERTZ, R. 1945 The quantitative relationship between stilbestrol response and dietary "folic acid" in the chick. Endrocrinology, *37:* 1.

———— 1948a The role of factors of the B-complex in estrogen metabolism. Recent Progress in Hormone Research, *2:* 161.

———— 1948b Dietary impairment of estrogen response in the immature monkey. Proc. Soc. Exp. Biol. Med., *67:* 113.

———— 1948c Interference with estrogen-induced tissue growth in the chick genital tract by a folic acid antagonist. Science, *107:* 300.

HERTZ, R., AND W. H. SEBRELL 1944 Impairment of response to stilbestrol in the oviduct of chicks deficient in the L. casei factor "folic acid." Ibid., *100:* 293.

NELSON, M. M., AND H. M. EVANS 1946 Pantothenic acid deficiency and reproduction in the rat. J. Nutrition, *31:* 497.

———— 1947 Reproduction in the rat on purified diets containing succinylsulfathiazole. Proc. Soc. Exp. Biol. Med., *66:* 289.

———— 1948 Effect of desoxypyridoxine on reproduction in the rat. Ibid., *68:* 274.

WELCH, A. D., R. W. HEINLE, G. SHARPE, W. L. GEORGE AND M. EPSTEIN 1947 Chemical antagonism of pteroylglutamic acid in a pig; hematopoietic effect of extrinsic and intrinsic factors. Ibid., *65:* 364.

THE MICROBIOLOGICAL ASSAY OF "FOLIC ACID" AS APPLIED TO MILK

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(Received for publication November 26, 1948)

The folic acid content of cow's milk whey was reported by Wright, McMahan, Cheldelin, Taylor, Snell and Williams in 1941. Their data suggested that milk contained only a small amount of folic acid. Microbiological data published by Williams, Cheldelin and Mitchell ('42); by Wright, Skeggs, Welch, Sprague and Mattis ('45); and by Luckey, Briggs, Moore, Elvehjem and Hart ('45); and rat data obtained by Day, Wakin, Zimmerman and McClung ('46) support this suggestion. However, precise data for a number of milk samples have not been presented, the suggestion of Wright et al. ('45) that milk contains a large amount of "potential folic acid" has not been verified, and data concerning the lability of "folic acid" during the heat processing of milk have not been obtained. An investigation originally planned to clarify these three points developed into a critical study of the assay methods for "folic acid" as applied to milk. The results of that study are presented here.

EXPERIMENTAL AND RESULTS

Methods and reagents

Investigations in our laboratory support the theory that milk has a relatively low content of "folic acid" and does not contain any large amounts of "potential folic acid." The results of these investigations do not establish the exact

amount of "folic acid" in milk, although approximately 20,000 tubes have been filled, inoculated and titrated in microbiological assays which were designed to contribute either directly or indirectly to answering this question. We have not found the available methods sufficiently precise or specific to establish completely the average "folic acid" content of milk. In our laboratory when these methods were applied to the assay for "folic acid" in milk both *L. casei* and *Streptococcus faecalis* (American Type Culture Collection no. 8043) gave erratic results which were not at all comparable in precision to those obtained when these organisms were used for the assay of other vitamins or amino acids. When applied to milk the *L. casei* results were in general much higher than those secured by the use of *S. faecalis*, but were more variable, showed a greater tendency to drift, and were less reproducible than the *S. faecalis* results. The methods used were those suggested by Teply and Elvehjem ('45). We prefer the titrimetric methods since they permit the use of unclarified extracts for assay purposes. Synthetic pteroylglutamic acid was used as a standard in all our "folic acid" studies. Hog kidney conjugase was prepared according to the method of Bird, Robbins, Vandenbelt and Pfiffner ('46). The conjugase preparation was kept frozen until a few minutes before use.

A crude chicken pancreas conjugase preparation was made by grinding chicken pancreas with an equal weight of water in a Waring Blendor and filtering with the aid of celite through a no. 42 Whatman filter. A clear filtrate was obtained for the first portion of the extract filtered and this was frozen and stored for use. Later portions of the filtrate were cloudy and could not be clarified. The cloudy filtrate was less active and possessed a higher blank value. It was discarded.

Sample preparation

In the preparation of liquid milk products for microbiological assay the simplest procedure is simply to dilute the sample so that the concentration of the unknown will fall in

the assay range. This is the procedure used for our first assays for "folic acid." In view of the well-known interference of fatty acids and other materials in unclarified extracts in the assay of riboflavin, pantothentic acid and biotin, we attempted to prepare clarified extracts of milk for "folic acid" assays. The casein in milk was precipitated by adjusting the pH to 4.5 with hydrochloric acid, the precipitated casein and occluded fat filtered off, an aliquot of the filtrate taken and brought

TABLE 1

Loss of "folic acid" during protein precipitation

SAMPLE	PRE-ASSAY SAMPLE TREATMENT	RECOVERY OF ADDED SYNTHETIC PTEROYLGLUTAMIC ACID	
		By *L. casei* method	By *S. faecalis* method
		%	%
Fresh milk	1. Straight dilution	91	88
	2. Proteins precipitated at pH 4.5 and at pH 7.0	85	58
	3. Proteins precipitated with hydrochloric and metaphosphoric acids at pH 7.0	0	31
Evaporated milk	1. Straight dilution	102	101
	2. Proteins precipitated at pH 4.5 and at pH 7.0	62	31
	3. Proteins precipitated with hydrochloric and metaphosphoric acids at pH 7.0	0	35

to pH 7.0 with sodium hydroxide, and a second filtration made through a no. 42 Whatman filter paper. While this filtration gave reasonably clear filtrates in most cases, one could increase the filtration rate and insure an entirely clear final filtrate by making the first precipitation with a mixture of metaphosphoric and hydrochloric acids. These procedures proved to be unsatisfactory, however, because when pteroylglutamic acid was added to the milk before the protein precipitation, recovery experiments indicated that large losses occurred (table 1). The pteroylglutamic acid is probably

lost by adsorption on the precipitated protein, but this has not been proved.

Since the same discrepancy in results of the *L. casei* and *S. faecalis* assays occurred with dry skim milk powder as with fresh, evaporated, or dried whole milk, it did not appear that the discrepancy could be caused by the presence of fat in the milk extract. Nevertheless, in order to check this point further the trace of fat which occurs in dry skim milk was removed by repeated extraction with diethyl ether. The assay results were similar before and after extraction, and prove that the presence of fat in the extracts is not the cause of the great discrepancy between the *L. casei* and *S. faecalis* results.

Conjugase investigations

A large number of assays were conducted after treatment of the milk samples with hog kidney conjugase. Preliminary treatments of milk samples with increasing amounts of hog kidney conjugase gave the same results for all increments and suggested that milk was free from inhibitors but that little or no "folic acid" conjugase was present in it. Later tests already reported (Hodson, '48) proved that hog kidney conjugase could be inhibited by protein and that for assay purposes the hog kidney conjugase treatment was of little value if carried out in the presence of relatively large amounts of protein.

Considerable trouble was experienced in arriving at optimum conditions for the hog kidney conjugase treatments. Since one of the problems encountered is common to other enzymatic treatments, it seems pertinent to discuss it briefly. This problem is that none of the commonly used preservatives prevented bacterial growth during the incubation period. The first treatment tried consisted of adjusting the pH of a diluted sample to 4.5, adding the desired quantity of the enzyme preparation, and incubating overnight at 45 or 37°C. The former temperature was used most often. After the overnight incubation the pH was adjusted to 6.8 and further di-

lution made if necessary. This procedure gave good recoveries and seemed satisfactory when applied to evaporated milk. With fresh or powdered milk samples it consistently gave erratic recoveries of added pteroylglutamic acid and results which were not reproducible. In an attempt to improve the enzyme treatment, fresh milk samples were incubated at pH 6.5–6.8 instead of 4.5 and at 37°C. Much higher values resulted from this change in pH. The samples were always curdled at the end of the overnight period but at first this was attributed to proteolytic enzymes in the hog kidney conjugase preparation.

This work was repeated and in the second trial some controls without enzyme but covered with toluene were incubated overnight at 37°C. and others were held overnight at 5°C. From the results of this trial, reported in table 2, it is apparent that covering the sample with toluene during the incubation did not prevent souring, and that bacterial synthesis of "folic acid" apparently takes place during the souring. It is possible that some bacterial growth and souring took place even in the samples held at 5°C. Bacterial growth and "folic acid" synthesis appear to have been limited at pH 4.5, but erratic recovery results in other experiments suggest this may not always be true when fresh milk is incubated with hog kidney conjugase for periods of several hours' duration. They suggest that the enzyme solution may introduce bacteria which multiply and increase the "folic acid" synthesis. Samples of high quality fresh milk (initial pH 6.6–6.8) have been treated with toluene, with benzene, with toluene and chloroform, and with benzene and chloroform and held overnight at 37°C. None of these preservatives prevented the souring of fresh milk. A preservative for this purpose should completely inhibit bacterial growth, should be easily inactivated or removed from the sample, should not react with "folic acid," should not react with or inactivate the conjugase, and should not react with other constituents of the sample or enzyme preparation to form products that will interfere in the enzymatic treatment or in the assay. A preservative

TABLE 2

Error produced by bacterial growth during an overnight conjugase incubation period

SAMPLE	TREATMENT	BACTERIAL GROWTH	"FOLIC ACID" BY S. FAECALIS ASSAY
			μg/l
A Fresh milk	1. Straight dilution held overnight at 5°C.	Suspected	5.5
	2. Incubated overnight at pH 4.5 without conjugase	Suspected	1.3
	3. Incubated overnight at pH 4.5 with conjugase	Suspected	1.0
	4. Incubated overnight at an initial pH of 6.6 without conjugase	Verified — milk soured and clabbered	21.7
	5. Incubated overnight at an initial pH of 6.6 with conjugase	Verified — milk soured and clabbered	more than 25.0
B Fresh milk	1. Straight dilution held overnight at 5°C.	Probably slight	1.0
	2. Incubated overnight at pH 4.5 without conjugase	Probably slight	0.5
	3. Incubated overnight at pH 4.5 with conjugase	Probably slight	0.6
	4. Incubated overnight at an initial pH of 6.6 without conjugase	Verified — milk soured and clabbered	8.6
	5. Incubated overnight at an initial pH of 6.6 with conjugase	Verified — milk soured and clabbered	17.6

meeting these requirements has not been found. The results reported in table 2 suggest the need for checking whether bacterial growth takes place during the incubation of other materials under toluene with conjugase, trypsin, and other enzymes.

Since a suitable preservative was not found, the procedure for enzymatic treatment was modified to minimize rather than eliminate bacterial growth. The length of the incubation period was reduced to one hour. A temperature of 45°C. was

TABLE 3

Lack of inhibitory effect of proteins on the activity of chicken pancreas conjugase

PROTEINS TESTED	"FOLIC ACID" IN YEAST	
	By *L. casei* assay	By *S. faecalis* assay
	μg/gm	*μg/gm*
Yeast	0 [1]	0 [1]
Yeast plus chicken pancreas conjugase	14,270	13,167
Yeast plus casein (1:100)	0 [1]	0 [1]
Yeast plus casein (1:100) plus chicken pancreas conjugase	15,963	14,600
Yeast plus gelatin (1:100)	0 [1]	0 [1]
Yeast plus gelatin (1:100) plus chicken pancreas conjugase	14,132	14,519

[1] Some free "folic acid" is present in the yeast but this was not detected at the assay level used. The free "folic acid" is a small fraction of the total "folic acid" in this yeast sample.

used since this is optimum for the conjugases and above optimum for many bacteria found in commercial samples of fresh milk. Samples were diluted, prepared for assay and autoclaved as soon as possible after incubation with the enzyme. The hog kidney conjugase treatments were made at a pH of 4.5, which is below optimum for many bacteria. The chicken pancreas conjugase treatments were made at a pH of 7–8, since this enzyme is inactive at pH 4.5.

After it had been shown that the hog kidney conjugase was unsuitable for the determination of "folic acid" conjugates in milk products, extensive tests and assays were conducted

with the crude chicken pancreas conjugase preparation described above. Mims, Swendseid and Bird ('47) report that the chicken pancreas conjugase is not inhibited by nucleic acid as is the hog kidney conjugase. The data given in table 3 demonstrate that the chicken pancreas conjugase is not inhibited by protein. To test further for the presence of inhibitors in milk, mixtures of yeast and evaporated milk and pteroyl-triglutamic acid and evaporated milk were tested with *S. faecalis* before and after incubation with chicken pancreas conjugase. Before enzyme treatment the yeast and milk

TABLE 4

Effect of chicken pancreas conjugase treatment on the "folic acid" content of evaporated milk

SAMPLE NUMBER	"FOLIC ACID" IN RECONSTITUTED MILK			
	L. casei method		*S. faecalis* method	
	Without conjugase	With conjugase	Without conjugase	With conjugase
	μg/gm	μg/gm	μg/gm	μg/gm
1	16	23	2.1	2.7
2	9	15	0.8	1.2
3	4	4	0.7	0.9
4	16	16	1.3	2.0
5	18	18	1.1	1.2
6	10	14	1.4	1.8
Average	12	13	1.2	1.6

mixture give a value of 1.72 μg per liter; after enzyme treatment the value increased to 7.75. Before enzyme treatment the pteroyl-triglutamic acid and milk mixture gave a value of 1.61; after conjugase treatment the value increased to 4.35 μg per liter. These data clearly illustrate that the chicken pancreas conjugase is active in the presence of milk. In table 4 data on the "folic acid" content of evaporated milk before and after conjugase treatment are given. These suggest that a small amount of "folic acid" conjugate may be present in some samples of milk, but since the methods are not highly precise they can hardly be considered to prove this point com-

pletely. In any case it appears that no large amounts of the conjugated forms of pteroylglutamic acid, such as are found in yeast, are present in milk. The chicken pancreas conjugase has been used to treat a considerable number of milk samples since this treatment may help to give a slightly better understanding of the "folic acid" content of milk as determined microbiologically than methods measuring only "free folic acid." In table 5 data on the "folic acid" content of milk are given. A more extensive study was made of the "folic acid" content of evaporated milk, since samples from all parts of the United States were available for assay.

TABLE 5

"Folic acid" content of milk

(Assayed after treatment with chicken pancreas conjugase)

TYPE OF SAMPLE	NUMBER OF SAMPLES	"FOLIC ACID" IN FRESH OR RECONSTITUTED MILK			
		L. casei assay		*S. faecalis* assay	
		Average	Range	Average	Range
		μg/gm		μg/gm	
Fresh milk	12	24	11–74	1.6	0.9–2.4
Evaporated milk	52	13	2–29	1.6	0.8–3.3
Dry skim milk	11	18	13–26	1.4	0.7–2.2

Sterilization losses

An investigation was made of whether or not there is a loss of "folic acid" activity during the sterilization of evaporated milk. Average results from 6 samples show no loss of "folic acid" activity as measured by the *S. faecalis* assay. Of the greater activity given by the *L. casei* assays, a loss of about 15% is shown. When 100 μg of synthetic pteroylglutamic acid per a 14½ oz. can were added to evaporated milk, approximately 100% recovery after sterilization was obtained with either the *S. faecalis* or *L. casei* assays. The results showing the stability of "folic acid" during the sterilization of evaporated milk are in contrast to the large losses of up to 97% found by Cheldelin, Woods and Williams ('43) to

occur during the cooking of many foods and to the losses of 54 to 92% found by Schweigert, Pollard and Elvehjem ('46) to occur during the cooking of meats. Although because of the wide difference in actual values obtained by the *L. casei* and *S. faecalis* methods it is rather difficult to evaluate fully the results on the unfortified samples of milk, the results on the fortified samples show that pteroylglutamic acid is not destroyed by the sterilization treatment. These results are supported by the data of Daniel and Kline ('47), who found that a solution of crystalline folic acid could be autoclaved for 30 min. at 121°C. and at a pH of 6.8 without significant loss.

Chick assay

Before the microbiological data compiled above were entirely collected it became apparent that it would not be easy to explain the discrepancy between the *L. casei* and *S. faecalis* results, and a small chick assay was planned to show whether or not any large amounts of "folic acid" might be present in conjugated or other forms available to the chick but not to bacteria. Shortly after the chick assay was completed, Lillie and Briggs ('47) published data obtained by a chick method showing that dry skim milk had a very low content of "folic acid," but their value is higher than is usually found with either microbiological method. When the writer recalculates their data to a reconstituted milk basis, a value of approximately 60 μg per liter is obtained. In our laboratory data in this range have, on a few occasions, been obtained with the *L. casei* method but never with the *S. faecalis* method.

The chick assay was performed by the method of Oleson, Hutchings and Sloane ('46). Ten chicks were fed the basal ration, and groups of 8 chicks each were fed each of the respective supplemented rations. The chicks were weighed weekly. At the end of the 4-week experiment hemoglobin determinations and feather and perosis scorings were made. The effect of pteroylglutamic acid on feathering has been shown by Oleson, Hutchings and Sloane ('46) and the effect

on perosis mentioned by Daniel, Farmer and Norris ('46). The anti-anemia effect was one of the first physiological properties of "folic acid" (vitamin Bc) noted by Pfiffner, Binkley, Bloom, Brown, Bird, Emmett, Hogan and O'Dell ('43).

The sample of dry whole milk was especially prepared for the assay. It received no preliminary concentration or heat treatment except pasteurization before spray drying. Levels of 10 and 20% of dry whole milk supplements were used.

TABLE 6

Chick assay for "folic acid" in dry whole milk powder

RATION	28-DAY DATA				
	Survival 8–28 days	Average weight	Average perosis score [1]	Average feathering score [2]	Average hemoglobin in blood
	%	*gm*			*gm/100 ml*
Basal	80	125	2.0	16	5.6
Basal + 0.15 mg pteroylglutamic acid per kg	86	186	2.5	30	6.3
Basal + 0.5 mg pteroylglutamic acid per kg	100	273	0.3	83	8.6
Basal + 1.0 mg pteroylglutamic acid per kg	100	286	0.3	79	9.6
Basal + 10% dry whole milk	100	142	2.4	14	6.3
Basal + 20% dry whole milk	75	137	2.3	14	5.6

[1] Zero indicates no perosis, 4 indicates slipped tendon.
[2] Zero indicates no feathers, 100 indicates normal feathering.

Higher levels tend to unbalance the ration so that the physiological response may no longer be comparable to that shown on the standard rations. The use of a level as high as 20% may be questionable. This is especially true in the case of dry milk products, which have a flushing effect on chicks.

The data presented in table 6 cannot easily be used to assign a definite value for the "folic acid" content of the sample

under assay. Although the chick method is not sensitive enough to assay milk, it would appear to give results higher than those given by either the *L. casei* or *S. faecalis* methods but which approach more closely to those of the former.

DISCUSSION

In this report the term "folic acid" has been used very loosely to include any response of the test organism. Although this response was measured by comparison to a standard response produced by synthetic pteroylglutamic acid, it is apparent that in milk we may also be measuring other active compounds, and that the biological response may be altered by the presence of inhibitors and synergists. The fact that milk contains other active compounds, inhibitors or synergists may explain the lack of agreement in results secured by the various methods, since the response of *L. casei*, *S. faecalis* and the chick to these substances is known to vary.

There are many compounds that show stimulatory, substitute, or inhibitory effects for *L. casei* and *S. faecalis*. Some of these are not known to occur in nature, and the presence of any of them in significant quantities in milk has not been established. However, the presence of compounds of this type may well explain the discrepancy in results secured when the three methods under discussion are applied to milk. Stokes ('44) has discussed the folic acid activity of thymine and its nucleoside, thymidine. Krueger and Peterson ('45) have studied the response of *L. casei* and *S. faecalis* to thymine. Synthetic inhibitors have been discussed by Daniel, Norris, Scott and Heuser ('47); by Daniel and Norris ('47); by Franklin, Stokstad and Jukes ('47); by Hutchings, Mowat, Oleson, Stokstad, Boothe, Waller, Angier, Semb and Subbarow ('47); by Wooley and Pringle ('48); by Oleson, Hutchings and Subbarow ('48); by Hitchings, Elion, Vander Werff and Falco ('48), and by Strandskov and Wyss ('46). Colio and Babb ('48) reported on a stimulatory growth factor for *S. lactis R (S. faecalis ?)*. Daniel, Scott, Heuser and Norris ('48) have

reported a factor stimulating *L. casei.* Daniel, Scott, Norris and Heuser ('48) have provided information on the physiological activity of many synthetic pteridines and pyrimidines for chicks.

The reports of Shorb ('48), West ('48) and Rickes, Brink, Koniuszy, Wood and Folkers ('48) on vitamin B_{12} may not be directly related to interference in "folic acid" assays, but they do point out the similar effect of vitamin B_{12} and pteroylglutamic acid in promoting hemoglobin regeneration in human anemias. However, vitamin B_{12} appears to be a more specific agent for the alleviation of Addisonian pernicious anemia. Ott, Rickes and Wood ('48) have reported that vitamin B_{12} stimulates chick growth on a diet low in the animal protein factor.

It is thus obvious that there are a large number of compounds with physiological properties which overlap or counteract those of pteroylglutamic acid. Since the response to these compounds varies from organism to organism, it is obvious that it is difficult if not impossible to assess accurately the "folic acid" activity of all foods and feeds by comparison with a single standard such as pteroylglutamic acid. Some foods can be accurately assayed, others cannot. It is our experience that milk falls in the latter class.

SUMMARY

Data are presented which demonstrate that with the methods at present available it is difficult to arrive at an accurate estimate of the average "folic acid" content of milk.

Data secured by two microbiological methods and a chick technic are not in agreement, but those obtained by any of the methods support the conclusion reached by other investigators, that milk is a poor source of "folic acid."

ACKNOWLEDGMENTS

The author wishes to thank the officials of the Pet Milk Company for releasing this report for publication. He especially thanks Dr. E. A. Louder, Technical Director, for his part in this connection as well as his supervision of the research

program. The author also thanks Dr. E. L. R. Stokstad of the Lederle Laboratories for supplying the pteroylglutamic acid and the pteroyl-triglutamic acid, and acknowledges the laboratory assistance of Roberta Biggins.

LITERATURE CITED

BIRD, O. D., MARY ROBBINS, J. M. VANDENBELT AND J. J. PFIFFNER 1946 Observation on vitamin Bc conjugase from hog kidney. J. Biol. Chem., *163:* 649.

CHELDELIN, VERNON H., ALTHEA M. WOODS AND ROGER J. WILLIAMS 1943 Losses of B vitamins due to cooking of foods. J. Nutrition, *26:* 477.

COLIO, LLOYD G., AND VIRGINIA BABB 1948 Study of a new stimulatory growth factor. J. Biol. Chem., *174:* 405.

DANIEL, ESTHER P., AND O. L. KLINE 1947 Factors affecting folic acid determination. Ibid., *170:* 739.

DANIEL, LOUISE J., FLORENCE A. FARMER AND L. C. NORRIS 1946 Folic acid and perosis. Ibid., *163:* 349.

DANIEL, LOUISE J., AND L. C. NORRIS 1947 Growth inhibition of bacteria by synthetic pterins. II. Studies with *Escherichia coli, Staphylococcus aureus,* and *Lactobacillus arabinosus* showing synergism between pterin and sulfonamide. Ibid., *170:* 747.

DANIEL, LOUISE J., L. C. NORRIS, M. L. SCOTT AND G. F. HEUSER 1947 Growth inhibition of bacteria by synthetic pterins. I. Studies with *Streptococcus faecalis, Lactobacillus casei* and *Lactobacillus arabinosus.* Ibid., *169:* 689.

DANIEL, LOUISE J., M. L. SCOTT, G. F. HEUSER AND L. C. NORRIS 1948 Further studies of unidentified factors required in the nutrition of *Lactobacillus casei.* Ibid., *174:* 71.

DANIEL, LOUISE J., M. L. SCOTT, L. C. NORRIS AND G. F. HEUSER 1948 The effect of synthetic pterins on growth and hemoglobin formation in the chick. Ibid., *173:* 123.

DAY, HARRY G., K. G. WAKIM, WILLIAM H. ZIMMERMAN AND L. S. MCCLUNG 1946 Nutritional status of rats on milk diets containing succinylsulfathiazole. J. Nutrition, *31:* 355.

FRANKLIN, A. L., E. L. R. STOKSTAD AND T. H. JUKES 1947 Acceleration of pteroylglutamic acid deficiency in mice and chicks by a chemical antagonist. Proc. Soc. Exp. Biol. Med., *65:* 368.

HITCHINGS, GEORGE H., GERTRUDE B. ELION, HENRY VANDER WERFF AND ELVIRA FALCO 1948 Pyrimidine derivatives as antagonists of pteroylglutamic acid. J. Biol. Chem., *174:* 765.

HODSON, A. Z. 1948 Inhibitory effect of pure and semipurified proteins on the activity of hog kidney conjugase. Arch. Biochem., *16:* 309.

HUTCHINGS, B. L., J. H. MOWAT, J. J. OLESON, E. L. R. STOKSTAD, J. H. BOOTHE, C. W. WALLER, R. B. ANGIER, J. SEMB AND Y. SUBBAROW 1947 Pteroylaspartic acid an antagonist for pteroylglutamic acid. J. Biol. Chem., *170:* 323.

KRUEGER, KEATHA, AND W. H. PETERSON 1945 The response of *Lactobacillus casei* and *Streptococcus faecalis* to vitamin Bc and thymine. Ibid., *158:* 145.

LILLIE, ROBERT J., AND GEORGE M. BRIGGS 1947 Biological assay of folic acid activity in common feedstuffs. Poultry Sci., *26:* 289.

LUCKEY, T. D., G. M. BRIGGS, JR., P. R. MOORE, C. A. ELVEHJEM AND E B. HART 1945 Studies on the liberation of compounds in the folic acid group. J. Biol. Chem., *161:* 395.

MIMS, VIRGINIA, MARIAN E. SWENDSEID AND O. D. BIRD 1947 The inhibition of pteroylglutamic acid conjugase and its reversal. The effect of nucleic acid and sulfhydryl combining reagents. Ibid., *170:* 367.

OLESON, J. J., B. L. HUTCHINGS AND N. H. SLOANE 1946 The effect of the synthetic pteroylglutamic acid on the feathering of chicks. Ibid., *165:* 371.

OLESON, J. J., B. L. HUTCHINGS AND Y. SUBBAROW 1948 Studies on the inhibitory nature of 4-aminopteroylglutamic acid. Ibid., *175:* 359.

OTT, WALTHER H., EDWARD L. RICKES AND THOMAS R. WOOD 1948 Activity of crystalline vitamin B_{12} for chick growth. Ibid., *174:* 1047.

PFIFFNER, J. J., S. B. BINKLEY, E. S. BLOOM, R. A. BROWN, O. D. BIRD, A. D. EMMETT, A. G. HOGAN AND B. L. O'DELL 1943 Isolation of the anti-anemia factor (vitamin Bc) in crystalline form from liver. Science, *97:* 404.

RICKES, EDWARD L., NORMAN G. BRINK, FRANK R. KONIUSZY, THOMAS R. WOOD AND KARL FOLKERS 1948 Crystalline vitamin B_{12}. Ibid., *107:* 396.

SCHWEIGERT, B. S., ANN E. POLLARD AND C. A. ELVEHJEM 1946 The folic acid content of meats and the retention of this vitamin during cooking. Arch. Biochem., *10:* 107.

SHORB, MARY S. 1948 Activity of vitamin B_{12} for the growth of *Lactobacillus lactis*. Science, *107:* 397.

STOKES, J. L. 1944 Substitution of thymine for "folic acid" in the nutrition of lactic acid bacteria. J. Bact., *48:* 201.

STRANDSKOV, FREDE, AND ORVILLE WYSS 1946 The inhibtion of bacteria by thiopyrimidines. Ibid., *52:* 575.

TEPLY, L. J., AND C. A. ELVEHJEM 1945 The titrimetric determination of "*Lactobacillus casei factor*" and "*folic acid.*" J. Biol. Chem., *157:* 303.

WEST, RANDOLPH 1948 Activity of vitamin B_{12} in addisonian pernicious anemia. Science, *107:* 398.

WILLIAMS, R. J., V. H. CHELDELIN AND H. C. MITCHELL 1942 Studies on the vitamin content of tissue. II. University of Texas Publication 4237, p. 97.

WOOLEY, D. W., AND A. PRINGLE 1948 Some structural analogues antagonistic to pteroylglutamic acid. J. Biol. Chem., *174:* 327.

WRIGHT, LEMUEL D., J. RAYMOND MCMAHAN, VERNON H. CHELDELIN, ALFRED TAYLOR, ESMOND E. SNELL AND ROGER J. WILLIAMS 1941 Studies on the vitamin content of tissues. I. University of Texas Publication No. 4137, p. 46.

WRIGHT, LEMUEL D., HELEN R. SKEGGS, ARNOLD D. WELCH, KENNETH L. SPRAGUE AND PAUL A. MATTIS 1945 The existence of a micro-biologically inactive "folic acid"-like material possessing vitamin activity in the rat. J. Nutrition, *29:* 289.

THE EFFECTS OF THIOURACIL ON METABOLISM [1]

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(Received for publication December 20, 1948)

Articles appearing recently in scientific journals indicate that under certain conditions of control, including the relative maturity of the experimental subject, there may occur definite increases in economy of body gain and improvement in carcass quality of swine and poultry when small amounts of thiouracil are included in the ration (Turner et al., '44; Muhrer and Hogan, '45; Kempster and Turner, '45; Andrews and Schnetzler, '46; Muhrer et al., '47; Van Der Noot et al., '47, '48). Andrews and associates ('47) found little or no effect on the growth of lambs but noted a trend toward a higher grade of carcass. A report by Beeson et al. ('47b) dealing with swine notes only loss of appetite and myxedema when the thiouracil was added to the diet. Acevedo et al. ('48) report inferior gains resulting from the use of 0.25% thiouracil in the ration of growing swine fed ad libitum. Beeson and associates ('47a), working with steers, found that feed consumption was not affected and that there was a "tendency" for the thiouracil-fed animals to require less feed per pound of gain.

A reduction of 22% in CO_2 production in rats has been reported by Reineke and associates ('44), who supplied 0.1% thiouracil in drinking water. However, since their animals were fed ad libitum (personal communication), the CO_2 produced may have been a reflection of the amount of food consumed as well as of decreased metabolism caused by the drug.

[1] Authorized for publication on October 5, 1948 as paper no. 1477 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

Even with equalized feed intake, greater storage of fat must be accompanied by a considerably higher respiratory quotient, making CO_2 comparisons of little significance.

A study of these somewhat conflicting reports makes it appear that results are directly dependent on the level at which thiouracil is fed, the length of time during which it is given, and the age or stage of maturity of the experimental subject. It may be reasonably assumed from the evidence at hand that thiouracil should not be given to young rapidly growing animals, nor should it be fed for any extended period of time. It is of course known that the drug inhibits the function of the thyroid gland, and it is assumed that the efficiency of body gain results from a lowered basal metabolic rate and lesser activity. No reports have appeared, however, so far as the present authors are aware, in which actual measurements of body gains of fat, protein, and heat production have been made. The main objective of the work here reported was to establish the facts regarding the metabolic effects of thiouracil on the distribution of feed energy. A fundamental study of such a nature should be important as a background if thiouracil should come to have an important place in the economy of animal production.

EXPERIMENTAL

Three diets were prepared from a standard stock colony diet [2] in which the level of thiouracil [3] was the only variable. To the first, designated as the basal diet, none of the drug was added; to the second, 0.1% of thiouracil was added, and to the third, 0.2%. These diets were then compared in a 10-week growth, metabolism and body balance study. The experimental subjects were 24 male rats, selected as 8 sets of litter-mate triplicates. Each rat of a triplicate was assigned to one of the dietary groups, thus making three groups of 8 rats each. At the start of the experiment all rats were be-

[2] Rockland rat diet.

[3] The thiouracil (Deravet) was kindly furnished by the Lederle Laboratories, Pearl River, New York, through the courtesy of Dr. Mark Welsh.

tween 50 and 55 days of age with an average live weight of 184 gm; at this time one other animal from each litter was sacrificed in order to obtain a group of control rats of similar weight and condition, whose bodies were analyzed and the results used as a basis for calculating the composition of the experimental rats at the start of the experiment.

The feed intake was controlled by the paired method except for a period of 10 days early in the course of the experiment, when an attempt was made to establish the animals on a constant feed intake of 14.0 gm per day, divided into two equal portions and fed twice daily. It was hoped that this method of feeding would permit respiration measurements to be made on all individuals of a triplicate immediately following the consumption of allotted portions of feed. However, the thiouracil-fed animals failed consistently to consume all of their feed as quickly as the animals which received the basal diet. Thus it was impossibe to carry out this particular phase of the experiment as was originally planned. However, there was no interruption in the equalization of the daily feed intakes among the three rats of each triplicate.

The experimental procedure used was that described, in detail, in an earlier paper by Swift et al. ('34), except that in the drying of the rat bodies a somewhat different procedure was followed. After the contents of the alimentary tract had been removed and discarded, the bodies were placed in the freezing room and maintained at a temperature of −10°F., where they were quickly frozen. While the bodies were still in a frozen condition they were chopped into small pieces with a sharp hatchet. The chopping operation was done on a small block fitted with high side-boards on three sides to prevent any scattering of the choopped, frozen pieces of the rat bodies. The above operation was carried out in the freezing room, which practically eliminated the loss of any blood or pieces of body tissues due to their adherence to the cutting tool. The chopped, frozen rat bodies were dried in vacuum desiccators over concentrated H_2SO_4 at room temperature for a period of two weeks. The bodies were then weighed and ex-

tracted with ether for a 48-hour period. The treatment of the ether extract and the body residue was as previously described. When the rats were killed at the end of the experiment, the thyroid glands were removed and weighed and the bodies were analyzed for moisture, ether extract, nitrogen, and energy. The total heat production for the 10-week period was computed by the body balance procedure; that is, as the difference between the gross energy of the food and the sum of the energy of body increase and of excreta.

During the course of the experiment, between the 4th and 6th weeks, measurements of the fasting heat production of all animals were made by the Haldane procedure. The animals were fasted for a 24-hour period immediately prior to the time when they were placed in the chambers where the respiration measurements were made during a period of at least 7 hours.

The energy content of the three diets was determined, by analysis, to be 4,037 cal. per gram, the nitrogen content 3.95%, and the moisture content, 8.39%.

RESULTS

During the course of the experiment all of the refusals of feed were by the rats which received the thiouracil-supplemented diets. None of these refusals, however, occurred during the first 10 days of the experiment, thus making it apparent that the taste of thiouracil was not objectionable. Acevedo and associates ('48) recently reported a simliar observation in working with swine. They noted that the thiouracil-treated group consumed feed in amounts similar to those of the control group during the first 10 days of the feeding trial and also that there was no apparent difference between the rates of gain of the two groups during the first 9 to 18 days.

While Andrews and associates ('47) have reported that swine, sheep and cattle apparently object to the taste of thiouracil, which results in decreased food intake, the authors of this paper believe that the taste of thiouracil is only of secondary importance when it is fed to rats. Of primary

importance is the fact that the feeding of thiouracil brings about a lowering of the animal's metabolism, and one of the first external manifestations of this condition is a depression of appetite.

In the experiment reported here the equalized food intake was sufficient to produce some growth and to permit valid comparisons of food utilization.

The average gains in body weight (table 1), excluding the contents of the alimentary tract, were 38.2 gm for the control rats, 57.0 gm for the rats which received 0.1% thiouracil, and 53.9 gm for the rats which received 0.2% thiouracil. Statistical treatment reveals that these differences are significant, with odds of 87 to 1 and 26 to 1, respectively.

TABLE 1

Feed consumption, gain in body weight[1] *of rats, and dry matter of body gain*[2]

RATION	FOOD EATEN (DRY MATTER)	GAIN IN BODY WEIGHT	DRY MATTER OF BODY GAIN
	gm	*gm*	*gm*
Basal	904.5	38.2	19.6
Basal + 0.1% thiouracil	904.5	57.0	28.6
Basal + 0.2% thiouracil	904.5	53.9	33.8

[1] Excluding contents of alimentary tract.

[2] Each datum represents the average of 8 rats during a period of 70 days.

The average values for dry matter of body gain were 19.6 gm for the control rats, 28.6 gm for the rats which received 0.1% thiouracil, and 33.8 gm for the rats which received 0.2% thiouracil. The odds that these differences are significant are 737 to 1 and 637 to 1, respectively.

The greater significance obtained by the statistical treatment of dry matter of the body gains as compared to a similar treatment of the gains in body weight can be attributed directly to differences in moisture content of the rat bodies. The average moisture content was 66.2% for the control group, 64.7% for the 0.1% thiouracil group, and 61.7% for the 0.2% thiouracil group. The significance of the difference between the first two groups is expressed by odds

of 14 to 1, while the odds are 808 to 1 in the case of the first and third groups. The foregoing results demonstrate clearly that a consideration of gain in body weight without regard to possible variations in moisture content of the animal body may frequently lead to erroneous conclusions in the interpretation of the final results. In contrast to our results is the finding by Muhrer et al. ('47) that the carcasses of swine

TABLE 2

Distribution of feed nitrogen[1]

RATION	FEED	FECES	DIGESTED	BODY GAIN
	gm	*gm*	%	*gm*
Basal	39.0	10.0	74.4	1.72
Basal + 0.1% thiouracil	39.0	9.5	75.8	1.84
Basal + 0.2% thiouracil	39.0	9.7	75.3	1.51

[1] Each datum represents the average of 8 rats during a period of 70 days.

TABLE 3

Distribution of feed energy[1]

RATION	FEED ENERGY	FECES	DIGESTED	BODY GAIN			METABOLIZABLE	HEAT PRODUCTION
				Total	Protein	Fat		
	Cal.	*Cal.*	%	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*
Basal	3896.0	1140.9	71.4	116.7	58.7	58.0	2654.0	2537.3
Basal + 0.1% thiouracil	3896.0	1119.5	71.9	196.2	62.9	133.3	2667.5	2471.4
Basal + 0.2% thiouracil	3896.0	1117.6	72.0	261.8	51.5	210.3	2667.2	2405.4

[1] Each datum represents the average of 8 rats during a period of 70 days.

which had received thiouracil contained 3% more water than did those of their controls.

The addition of thiouracil to the diet did not affect its metabolizable energy nor the digestibility of its nitrogen or the feed energy. The average digestibility of nitrogen (table 2) was 74.4%, 75.8% and 75.3%, and of the feed energy (table 3) 71.4%, 71.9% and 72.0% with the control, 0.1% thiouracil, and 0.2% thiouracil rats, respectively.

Concomitant with a decrease in heat production, the rats receiving thiouracil stored more energy than the control group. The average energy gains (table 3) were 116.7 Cal. for the controls, 196.2 Cal. for the rats which received 0.1% thiouracil, and 261.8 Cal. for the rats which recceived 0.2% thiouracil. The odds that the difference is significant between the controls and the rats on the lowest level of thiouracil are 575 to 1, and they are 2,499 to 1 that the difference is significant between the controls and the rats which received thiouracil at the 0.2% level.

The differences in energy gained as protein are not significant, while the differences in energy gained as fat are

TABLE 4

Percentage distribution of feed energy and of metabolizable energy[1]

RATION	FEED ENERGY		METABOLIZABLE ENERGY	
	Body gain	Heat production	Body gain	Heat production
	%	%	%	%
Basal	2.9	63.6	4.4	95.6
Basal + 0.1% thiouracil	4.9	62.0	7.4	92.6
Basal + 0.2% thiouracil	6.6	60.3	9.8	90.2

[1] Each datum represents the average of 8 rats during a period of 70 days.

highly significant, the odds being 141 to 1 that the 0.1% thiouracil group gained more energy as fat than the control group, and 3,665 to 1 that the 0.2% thiouracil group gained more energy as fat than the control group. Of the energy retained by the control group, 58.8 Cal. were gained as protein and 57.9 Cal. as fat; with the group which received 0.1% thiouracil, 62.9 Cal. were gained as protein and 133.3 Cal. as fat; and with the 0.2% thiouracil group, 51.5 Cal. were gained as protein and 210.3 Cal. as fat.

The percentages of metabolizable energy utilized for body gain (table 4) were 4.4% for the controls, 7.4% for the 0.1% thiouracil, and 9.8% for the 0.2% thiouracil groups.

The groups of rats which gained the most energy (the rats which received thiouracil) produced less heat than the con-

trol group, the three groups of animals metabolizing essentially the same quantities of food energy. The determined values for the average total heat production for the 10-week experimental period were: 2537.3 Cal. for the control group, 2471.4 Cal. for the 0.1% thiouracil group and 2405.4 Cal. for the 0.2% thiouracil group, or 65.1%, 63.4% and 61.7% of the food energy, respectively. The statistical significance of the difference between the first two groups is characterized by odds of 21 to 1, while odds of 999 to 1 indicate the significance of the difference between the control group and the group which received thiouracil at the 0.2% level.

Consideration of the relative magnitudes of the values shown in this table makes clear the fact that at any plane of nutrition the energy represented by heat production is always far greater than that represented by gain of body substance. Consequently, a given percentage reduction in heat production, if accompanied by a similar absolute gain in body tissue, signifies a much larger percentage increase in the latter.

In regard to the fasting heat production, calculated on an hourly basis and corrected to uniform live weight (200 gm), the differences between the control and thiouracil treated animals were even more significant than in the case of the total heat production for the entire 70-day feeding experiment. The average hourly fasting heat production for the control group was 758 Cal.; for the group which received 0.1% thiouracil, 673 Cal.; and for the group which received 0.2% thiouracil, 634 Cal. The odds are 737 to 1 that the control group had a higher fasting heat production than the group which received thiouracil at the 0.1% level, and 4,999 to 1 that the former group had a higher fasting heat production than their pair mates which received the diet containing 0.2% thiouracil. The control group produced 12.6% and 18.0% more heat than the 0.1% and 0.2% thiouracil groups, respectively, the corresponding values for the 10-week feeding experiment being 2.7% and 5.5%.

There were significant differences in the average weights of the thyroid glands at the conclusion of the experiment,

the average weight in milligrams for the controls, 0.1% thiouracil and 0.2% thiouracil animals being respectively as follows: 10.4 ± 1.00, 34.7 ± 1.49 and 44.7 ± 3.18. No histological examination was made of the glands, and the general appearance of the livers, lungs, hearts, and kidneys was normal.

The fact that the heat production of the fasting period was more significantly decreased by thiouracil than was the heat production of the feeding period suggests that this drug brings about increased economy of food utilization through a reduction in basal metabolism (and probably muscular activity) and that the true dynamic effect of the ration may be entirely unaffected.

SUMMARY

Three diets in which the level of thiouracil was the only variable were compared in a 10-week growth, metabolism, and body balance study using 24 male rats, selected as 8 sets of litter-mate triplicates, which were between 50 and 55 days of age when placed on experiment. Each rat of a given triplicate received identical quantities of food. The metabolizable energy of the ration was unaffected by thiouracil and no significant differences were found in the digestibility of protein or feed energy or in the body gains of protein. However, the rats receiving 0.1% and 0.2% thiouracil in their diets gained respectively 1.7 and 2.2 times as much energy as the rats on the same diet without thiouracil, these differences in body gain being mainly in the form of fat. Greater gain in body fat was accompanied by a corresponding decrease in heat production.

LITERATURE CITED

ACEVEDO, R., B. S. SCHWEIGERT, P. B. PEARSON AND F. I. DAHLBERG 1948 Effect of feeding thiouracil to swine on the rate of gain and weight of thyroid gland. J. Animal Sci., *7:* 214.

ANDREWS, F. N., W. M. BEESON, E. R. BARRICK AND CLAUDE HARPER 1947 The influence of thiouracil and thiourea on the growth and fattening of lambs. Ibid., *6:* 3.

ANDREWS, F. N., AND E. E. SCHNETZLER 1946 Influence of thiouracil on growth and fattening in broilers. Poultry Sci., *25:* 124.

BEESON, W. M., F. N. ANDREWS AND P. T. BROWN 1947a The effect of thiouracil on the growth and fattening of yearling steers. J. Animal Sci., *6:* 16.

BEESON, W. M., F. N. ANDREWS, H. L. WITZ AND T. W. PERRY 1947b The effect of thyroprotein and thiouracil on the growth and fattening of swine (abstract). Ibid., *6:* 482.

KEMPSTER, H. L., AND C. W. TURNER 1945 The effect of feeding thiouracil on the fleshing of New Hampshire broilers. Poultry Sci., *24:* 94.

MCMILLEN, W. N., E. P. REINEKE, L. J. BRATZLER AND M. J. FRANCES 1947 The effect of thiouracil on efficiency of gains and carcass quality in swine. J. Animal Sci., *6:* 305.

MUHRER, M. E., AND A. G. HOGAN 1945 Effect of thiouracil on growing swine. Proc. Soc. Exp. Biol. Med., *60:* 211.

MUHRER, M. E., D. R. WARNER, Z. PALMER AND A. G. HOGAN 1947 Effect of thiouracil and protamone on growing swine (abstract). J. Animal Sci., *6:* 489.

REINEKE, E. P., A. B. SCHULTZE AND C. W. TURNER 1944 Studies of thyroid physiology by use of thiourea and its derivatives. J. Dairy Sci., *27:* 643.

SWIFT, R. W., O. J. KAHLENBERG, LEROY VORIS AND E. B. FORBES 1934 The utilization of energy producing nutriment and protein as affected by individual nutrient deficiencies. I. The effects of cystine deficiency. J. Nutrition, *8:* 197.

TURNER, C. W., E. P. REINEKE AND A. B. SCHULTZE 1944 The use of thiourea in the fattening of meat animals (abstract). J. Animal Sci., *3:* 448.

VAN DER NOOT, G. W., R. P. REECE AND W. C. SKELLEY 1947 The effect of thiouracil in the ration of growing swine. Ibid., *6:* 12.

———— 1948 Effects of thyroprotein and of thiouracil alone and in sequence in the ration of swine. Ibid., *7:* 84.

SIMULTANEOUS SURVEYS OF FOOD CONSUMPTION IN VARIOUS CAMPS OF THE UNITED STATES ARMY [1]

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FOUR FIGURES

(Received for publication February 7, 1949)

It is the purpose of this paper to record the results of simultaneous food consumption surveys conducted in 44 Army messes during the period 22 to 28 May 1945, inclusive. These surveys were carried out in compliance with an Army directive (ASF Circular No. 167, '45), for the purpose of reviewing the food requirements of troops. The study, together with other data, was used to form a sound basis for dietary recommendations in the Zone of Interior. The messes were chosen to be representative of the Army in the United States, and ranged from those serving soldiers performing sedentary duties to those serving soldiers undergoing rigorous training. This made possible procurement, under centralized control and standard methods, of Army food consumption data throughout the Zone of Interior, and distribution curves of these data are based on representative samples of food consumption at a given time. The information contained herein will add further to the studies made by Murlin and Hilde-

[1] The opinions expressed in this paper are those of the authors and do not necessarily represent the official views of any governmental agency.

brandt ('19), and Howe, Mason and Dinsmore ('19) in World War I, and by Howe and Berryman ('45) in World War II.

METHODS

Each individual survey in the 44 messes was under the direct supervision of a Medical Department Nutrition Officer. These officers were scientific specialists originally commissioned directly from civilian life into the Army of the United

TABLE 1

Average quantities of food consumed in 44 Army messes during the period 22 to 28 May 1945

FOOD GROUPS	CONSUMED 1945	CONSUMED 1941–1943 [1]
	lb./man/day	*lb./man/day*
Meat, fish and poultry	0.834	0.909
Eggs	0.165	0.149
Milk and milk products (fluid milk equivalents)	1.260	1.016
Fats, butter and spreads	0.070	0.083
Fats, other	0.044	0.069
Sugar and syrups	0.280	0.262
Cereals and grain products	0.512	0.567
Beans, other legumes (dry), nuts (dry)	0.036	0.055
Vegetables, leafy green or yellow	0.302	0.359
Tomatoes	0.162	0.155
Citrus fruits	0.332	0.248
Potatoes	0.562	0.561
Vegetables, other than leafy green or yellow	0.248	0.252
Fruits, other than citrus	0.211	0.358
Fruits, dried	0.028	0.026

[1] Average food consumption in army messes as reported by Howe and Berryman ('45).

States, and had had considerable experience in conducting similar surveys prior to the beginning of this study. Since these data were obtained during the same 10-day period at all stations, and the Nutrition Officers conducting the surveys were operating in accordance with centralized instructions, it is felt that a high degree of comparability was obtained in the results.

Nutritional evaluation of the quantities of food consumed was carried out by the short method of evaluation of food groups described by Berryman and Chatfield ('43) and by Berryman and Howe ('44).

RESULTS AND DISCUSSION

Table 1 shows the average quantities of the various food groups consumed in the mess per man per day during the

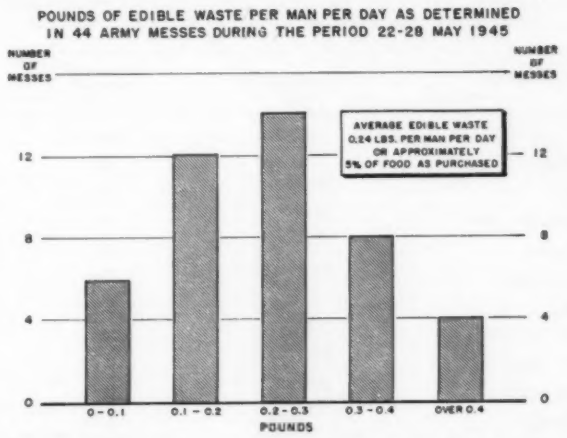
POUNDS OF EDIBLE WASTE PER MAN PER DAY AS DETERMINED
IN 44 ARMY MESSES DURING THE PERIOD 22-28 MAY 1945
NUMBER OF MESSES
NUMBER OF MESSES
AVERAGE EDIBLE WASTE 0.24 LBS. PER MAN PER DAY OR APPROXIMATELY 5% OF FOOD AS PURCHASED
12
8
4
0
0 - 0.1
0.1 - 0.2
0.2 - 0.3
0.3 - 0.4
OVER 0.4
POUNDS

Fig. 1 Distribution curve, showing edible waste during simultaneous surveys of 44 Army messes. Ordinates represent number of messes with a particular value of food waste; abscissae represent food wastage in pounds per man per day.

period under survey; for comparative purposes, food consumption figures as reported by Howe and Berryman ('45) are shown in column 2 of this table. In order to determine the nutritional adequacy of the food consumed it was necessary to estimate not only the amounts of food prepared but also the amount of each food item wasted, and to subtract the nutrients contained therein from the nutrients in the food prepared. To obtain data on food wastage, all plate waste was segregated by food groups and weighed, and all kitchen

waste was also segregated and weighed. The range of food wastage in the different messes as shown by our data in figure 1 was from 0.08 lb. to 0.66 lb. per man per day, resulting in an over-all average of 0.24 lb. of edible food per man per day or approximately 5% of the food issued by the Quartermaster. This figure, as compared to an average edible food waste of 0.38 lb. per man per day reported in 1919, and of

TABLE 2

Calculated nutritive value of average food consumed per man per day as determined in 44 Army messes during the period 22 to 28 May 1945

NUTRITIVE FACTORS	CONSUMED	STANDARD DEVIATION	NRC [1]
Calories	3,744	299	3,000
Protein (gm)	127	11	70
Fat (gm)	181	28	
Carbohydrates (gm)	411	54	
Calcium (gm)	1.3	0.2	0.8
Phosphorus (gm)	2.2	0.2	
Iron (mg)	23.3	2.1	12.0
Vitamin A (I.U.)	10,042	1,960	5,000
Thiamine (mg)	1.6	0.2	1.5
Riboflavin (mg)	2.9	0.4	2.0
Niacin (mg)	23.0	2.4	15.0
Ascorbic acid (mg)	89.0	12.6	75

[1] 1945 National Research Council recommended daily allowance for a moderately active man.

0.39 lb. in 1941 and 0.32 lb. in 1943 reported by Howe and Berryman ('45), indicates the creditable strides made by the Army on food conservation.

During this study data were also obtained on messing practices, i.e., total elapsed preparation times. The methods of cooking and preparation of food were satisfactory in the majority of mess halls. In some instances, however, improper preparation and poor cooking practices were employed, resulting in lowered nutritive value of the food consumed. The

most common infraction of good cooking practices was poor timing of food preparation: meat was cooked and salads prepared too far in advance. Another factor, also having a bearing upon the amount of food left over, and wasted, was the overdrawing and underdrawing of rations. A comparison was made of rations drawn with the number of men actually fed, based on head counts made by each Nutrition Officer, and

TABLE 3

Caloric value of food consumed by different types of organizations in 44 Army messes during the period 22 to 28 May 1945

TYPE OF UNIT	DEGREE OF DUTY ACTIVITY	MESSES SURVEYED	AVERAGE VALUE OF FOOD CONSUMED
		no.	*Cal./man/day*
Armored	Training	3	3,868
Engineer	Training	3	3,726
Field artillery	Training	5	3,722
Infantry	Training	15	3,828
Military police	Duty	3	3,828
Quartermaster	Training	2	4,078
Signal	Training	1	3,507
Transportation	Training	1	3,676
Miscellaneous			
Headquarters detachment	General duty	6	3,499
Hospital patient mess	Hospital	3	3,729
Basic training	Training	2	3,471

a compilation of the rations drawn. Of the messes surveyed, 79% over-drew rations and 20% under-drew rations. Overdrawing rations to the extent of 5% was the average for the 44 messes surveyed.

The approximate nutritive value of the food consumed for the period is shown in table 2. It will be noted that the quantities of essential nutrients as calculated are liberal, and exceed, in every instance, the National Research Council's recommended dietary allowances for moderately active men.

The various organizations and units surveyed were of widely different composition and included a broad range of duties and varying levels of physical activity. Included were artillery, cavalry, engineer, infantry, medical, military police, quartermaster, signal, tank transportation, replacement center and miscellaneous troop units. The average caloric value of food consumed by the various types of units, as shown by our data in table 3, ranged from 3,471 Cal. for two basic training units, to 4,078 Cal. for two quartermaster units, with a total average of 3,744 Cal. per man per day. These figures indicate there was no significant variation of caloric intake with degree of activity of troops. Our estimated average caloric intake (3,744) agrees closely with the estimate reported by Howe and Berryman ('45) in World War II (3,694) and the findings of Murlin and Hildebrandt ('19) in World War I (3,633). The observation by Howe and Berryman that the calculated caloric value of food consumed in the mess rarely lies outside of the range of 3,000 to 4,000 Cal. is corroborated by our data presented in table 3.

Distribution curves showing the calculated nutritive values of the average amount of food consumed per man per day during the study are shown in figures 2 to 4 inclusive.

By actual count, the total number of men served in all the messes during the period of survey is as follows: breakfast 77,441; dinner 85,185; and supper 72,344. It is interesting to note the disparity between the number of men served breakfast, dinner, and supper. Undoubtedly the mess-hall diet was

Fig. 2 Distribution curves, showing nutrients consumed during simultaneous survey of 44 Army messes. Upper left, *calories:* ordinates represent per cent of men with a particular value of caloric intake; abscissae represent calories consumed per man per day. Upper right, *proteins:* ordinates represent per cent of men with a particular value of protein intake; abscissae represent proteins in grams consumed per man per day. Lower left, *fat:* ordinates represent per cent of men with a particular value of fat intake; abscissae represent fat in grams consumed per man per day. Lower right, *carbohydrates:* ordinates represent per cent of men with a particular value of carbohydrate intake; abscissae represent carbohydrates in grams consumed per man per day.

NUTRIENTS CONSUMED PER MAN PER DAY AS DETERMINED IN 44 ARMY MESSES DURING THE PERIOD 22 – 28 MAY 1945

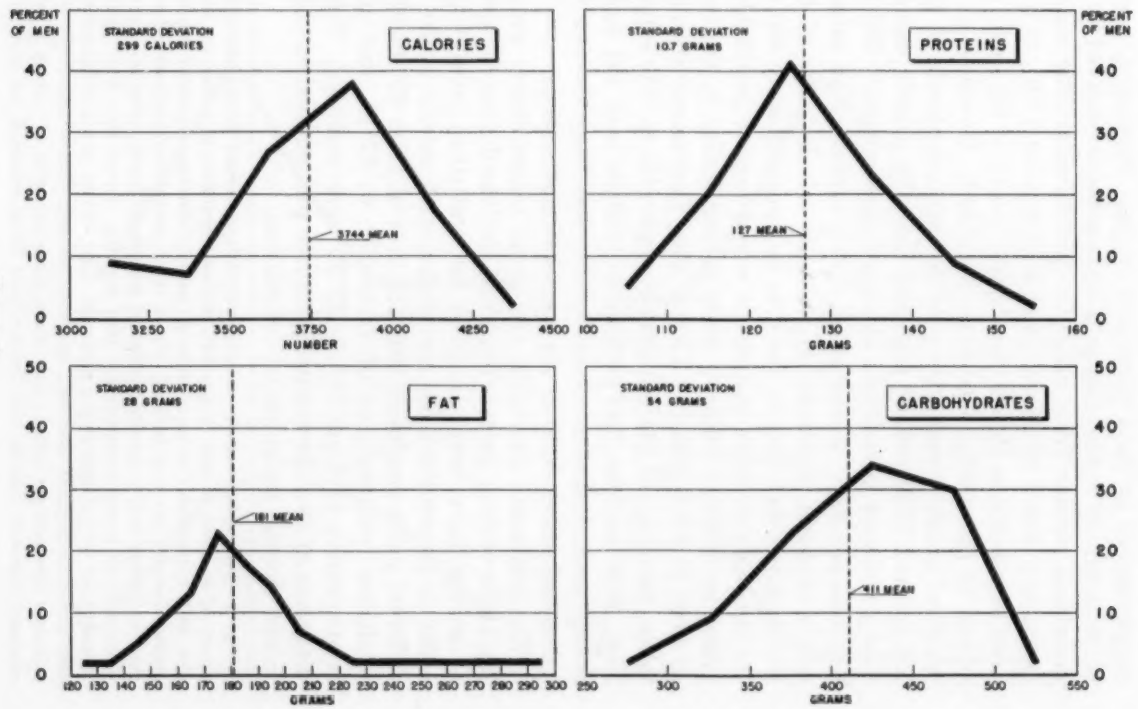

Figure 2

supplemented by food purchased at post exchanges, restaurants in nearby towns, and personal packages received by the individual soldier from relatives and friends. For reasons adequately listed by Murlin and Hildebrandt ('19), it is almost impossible to obtain an accurate record of the items consumed by the soldier outside the mess-hall. However, as the result of a nutritional survey of 261 canteens made in 1919, Murlin and Hildebrandt reported that the caloric value of food purchased at canteens averaged approximately 365 Cal. per man per day. McCay et al. ('44), reporting on sales by ship's canteens, estimate the average caloric value of food per man per day bought there to be approximately 400 Cal. On the basis of these findings it would appear justifiable to place the average soldier's daily caloric intake in the range of 4,000 to 4,100 Cal. i.e., 3,700 + 400. Cognizance should be taken, however, of the limitations applicable to this supposition. It is obvious that certain individuals may eat considerably more and others considerably less than the averages calculated above, both in the mess-hall and outside.

The minimum, maximum and mean temperatures prevailing at the various posts, camps and stations during the period of survey have been compiled for statistical treatment in order to determine their correlation with caloric intake. In different posts maximum temperatures of from 60° to 96°F., minimum temperatures of from 39° to 67°F., and mean temperatures of from 54° to 79°F. were recorded. Scatter diagrams of the various temperatures plotted against caloric intake showed no apparent correlation.

Fig. 3 Distribution curves, showing nutrients consumed during simultaneous survey of 44 Army messes. Upper left, *calcium:* ordinates represent per cent of men with a particular value of calcium intake; abscissae represent calcium in grams consumed per man per day. Upper right, *phosphorus:* ordinates represent per cent of men with a particular value of phosphorus intake; abscissae represent phosphorus in grams consumed per man per day. Lower left, *iron:* ordinates represent per cent of men with a particular value of iron intake; abscissae represent iron in milligrams consumed per man per day. Lower right, *vitamin A:* ordinates represent per cent of men with a particular value of vitamin A intake; abscissae represent vitamin A in International Units consumed per man per day.

NUTRIENTS CONSUMED PER MAN PER DAY AS DETERMINED
IN 44 ARMY MESSES DURING THE PERIOD 22 – 28 MAY 1945

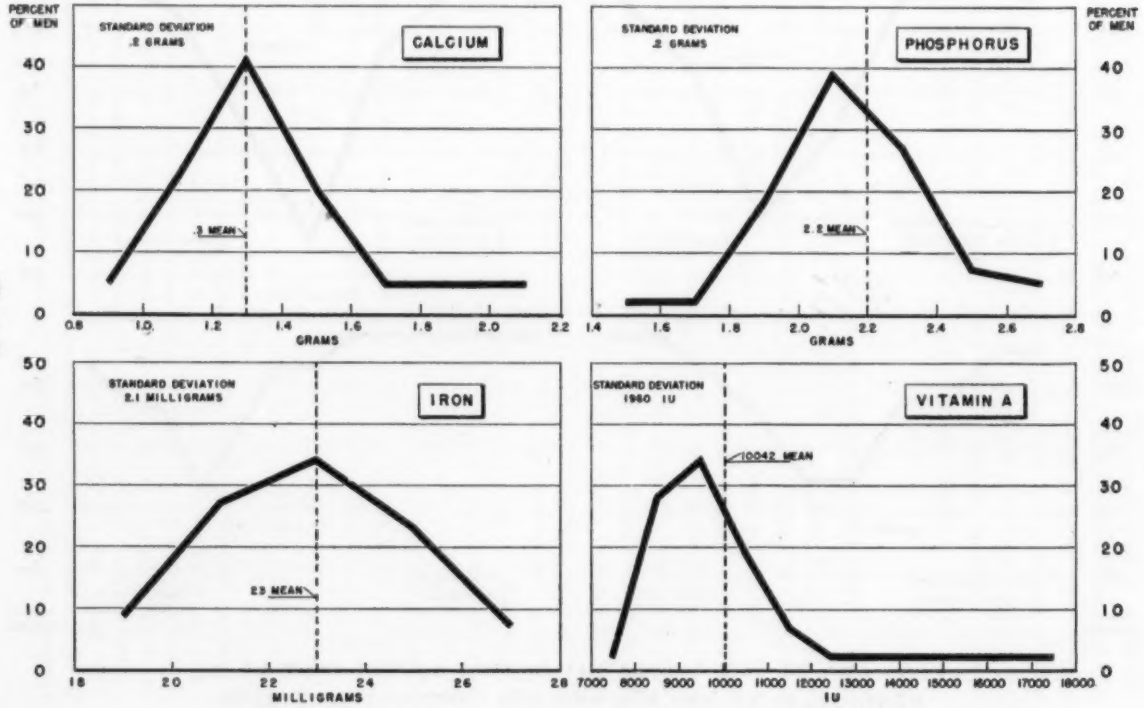

Figure 3

NUTRIENTS CONSUMED PER MAN PER DAY AS DETERMINED
IN 44 ARMY MESSES DURING THE PERIOD 22 – 28 MAY 1945

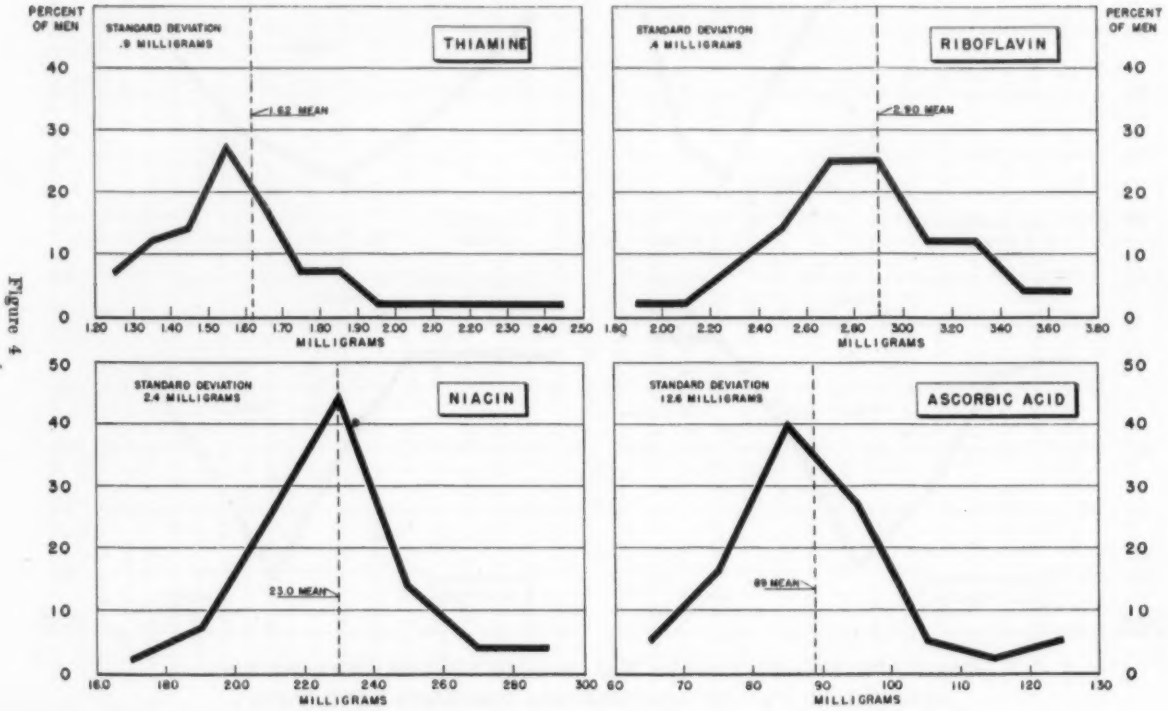

Figure 4

The 44 posts, camps and stations involved had a wide divergence of elevation, ranging from 10 ft. to 6,105 ft. above sea level. An attempt was made to determine a possible correlation between elevation and caloric intake. Elevations at the various posts were plotted against caloric intake at each post and analyzed statistically. In this particular instance no apparent correlation existed between these factors.

SUMMARY

Simultaneous nutrition surveys were carried out in May, 1945 at 44 military posts of the United States Army where Field Ration A, the rough equivalent of a liberal civilian diet in which there is a wide range of food, was provided. The food consumption levels reported indicated that, judged by National Research Council Standards, the American soldier in the Zone of Interior received a nutritionally adequate diet, although mess operations could have been improved through continued education and supervision of mess personnel. In the mess the average caloric intake of the average soldier per day, as determined by evaluation of food consumed, was approximately 3,700 Cal., fortified by an additional 300 to 400 Cal. consumed per day by virtue of food purchases at the post exchange. Food wastage comprised an average of 0.24 lb. of edible food per man per day. No significant correlations were found between the consumption of any individual nutrient and local mean temperature, altitude or the activity of the group.

Fig. 4 Distribution curves, showing nutrients consumed during simultaneous survey of 44 Army messes. Upper left, *thiamine:* ordinates represent per cent of men with a particular value of thiamine intake; abscissae represent thiamine in milligrams consumed per man per day. Upper right, *riboflavin:* ordinates represent per cent of men with a particular value of riboflavin intake; abscissae represent riboflavin in milligrams consumed per man per day. Lower left, *niacin:* ordinates represent per cent of men with a particular value of niacin intake; abscissae represent niacin in milligrams consumed per man per day. Lower right, *ascorbic acid:* ordinates represent per cent of men with a particular value of ascorbic acid intake; abscissae represent ascorbic acid in milligrams consumed per man per day.

ACKNOWLEDGMENTS

The food consumption values listed in this paper represent a summary of the data reported by and vouched for by Nutrition Officers carrying out nutritional surveys at various posts, camps and stations within this country. We wish to acknowledge the careful and diligent efforts of these officers which made this paper possible. Special acknowledgment is made to Captain Albert J. Dyer, under whose guidance the simultaneous surveys were so successfully completed.

Thanks are due to Dr. Robert E. Johnson, Director, Medical Nutrition Laboratory; Captain Denver I. Allen, Nutrition Branch, Surgeon General's Office; and Colonel Paul E. Howe, Nutrition Consultant, for their helpful suggestions and criticisms.

LITERATURE CITED

ASF CIRCULAR No. 167, 10 MAY 1945 Headquarters Army Service Force, Part Two, Section II: Nutrition Survey. To determine actual consumption of food by troops.

BERRYMAN, G. H., AND C. CHATFIELD 1943 A short method of calculating nutritive value of diets. J. Nutrition, *25:* 23.

BERRYMAN, G. H., AND P. E. HOWE 1944 A short method of calculating nutritive value of diets (concluded). Ibid., *27:* 231 (correction *27:* 434).

HOWE, P. E., AND G. H. BERRYMAN 1945 Average food consumption in training camps of the United States Army. Am. J. Physiol., *144:* 588.

HOWE, P. E., C. C. MASON AND S. C. DINSMORE 1919 Variations in strength and in the consumption of food by recruits and seasoned troops. Ibid., *49:* 557.

MCCAY, C. M., M. B. PINE, F. H. DAVIS, R. A. GORTNER, JR., G. P. HAUGEN AND J. H. SULLIVAN 1944 Nutrition Survey of the Mess at Naval Training Station, Norfolk, Virginia, 2–16 May 1944. Report from the Naval Medical Research Institute, Bethesda, Maryland (August, 1944).

MURLIN, J. R., AND F. M. HILDEBRANT 1919 Average food consumption in training camps of the United States Army. Am. J. Physiol., *49:* 531.

FOOD CONSUMPTION OF SOLDIERS IN A SUBARCTIC CLIMATE (FORT CHURCHILL, MANITOBA, CANADA, 1947–1948)[1]

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ONE FIGURE

(Received for publication February 7, 1949)

Little is known about the food consumption of garrison troops in arctic and subarctic climates. Most previous work has been done on troops in the field, such as in the Prince Albert Trials (Kark, Johnson and Lewis, '45) and in Exercise "Musk Ox" (Kark et al., '48). The present series of surveys was undertaken primarily to obtain information on the nutrient intake of garrison troops receiving an abundant ration in a subarctic climate. Secondary purposes were to obtain information on nutritional requirements as judged by medical criteria, and on possible peculiarities of food tastes in cold climates.

A favorable opportunity was offered at Fort Churchill, Canada, because the garrison troops in the winter of 1947–1948 were receiving an abundant and varied ration of fresh and frozen foods (Canadian Army Arctic Ration Scale 7, with an issue of 5,500 Cal. per man per day) and were under medical observation throughout the winter.

[1] The opinions expressed in this paper are those of the authors and do not necessarily represent the official views of any governmental agency.

Fort Churchill is situated on the west coast of Hudson Bay at the mouth of the Churchill River, about 500 miles south of the Arctic Circle. It was chosen for this survey because of the extreme "windchill" prevailing throughout the winter and early spring; in fact, it has one of the most consistently severe "windchills" of any inhabited area in the world and loss of body heat in exposed personnel is consequently very great. Although the temperature does not go as low as in

TABLE 1

Average winter weather conditions at Fort Churchill, Manitoba, Canada (10-year period, 1936–1946)

MEASUREMENT	MONTH				
	Nov.	Dec.	Jan.	Feb.	Mar.
Mean temperature (°F.)	+6	−11	−19	−17	−6
Mean wind velocity (m.p.h.)	17	15	15	15	14
Mean windchill[1] (kg cal./m²/hr.)	1,440	1,680	1,820	1,765	1,585
Total precipitation (inches, water equivalent)	1.03	0.66	0.48	0.61	0.87

[1] As defined by Siple ('45), "windchill," or dry shade atmospheric cooling, is a measure of the quantity of heat which the atmosphere is capable of absorbing from one square meter of exposed surface in one hour. It is expressed in kilogram calories per square meter per hour per degree Centigrade (0.369 B.T.U. per square foot per hour per degree Fahrenheit). The nomogram from which values are taken is based on the cooling rate upon a body at a neutral skin temperature of 33°C. (91.4°F.).

many places farther north, the average wind velocity is 15 to 20 miles per hour, and blizzards up to 50 miles per hour are quite common. Table 1 gives average winter conditions at Fort Churchill for the 10-year period from 1936 to 1946.

METHODS

In order to obtain data in early winter, mid-winter and late winter, surveys were conducted from the 9th to 18th of November, 1947; the 15th to 24th of February, 1948; and the 11th to 20th of April, 1948.

The surveys were conducted in a mess in which an average of 100 men ate all their meals. The military duties of these men included routine administration and general camp maintenance. Because of the great variety of duties of each man during the winter, it was impractical to secure a uniform record of activity. However, judging by interrogation and observation, the average man was moderately active and spent about three hours daily in the open.

In the conduct of the surveys, inventories were taken at the beginning and the end of each 10-day period, records were kept of food issued, plate waste and kitchen waste were weighed, and head counts were made at each meal. Computations were made by the short methods of Berryman and Chatfield ('43) and Berryman and Howe ('44), with the aid of the U. S. National Research Council's tables of food composition ('45).

At each meal a questionnaire listing all foods and beverages sold in the canteen (post exchange in U. S. Army terms) was given to 6 men at random; on this each man reported his consumption of these items for the previous day.

RESULTS AND DISCUSSION

Nutrient intake

The average intake of the various nutrients during the three 10-day surveys is shown in table 2. In every case the intake equals or exceeds the daily allowance recommended by the U. S. National Research Council for active men ('48). The reason for this excellent showing was because, on the average, the soldiers ate large amounts of a well-balanced ration. No specially supplemented foods were issued, and vitamin pills were not an item of issue.

This was an adequate diet from the medical viewpoint. Our staff at Fort Churchill kept continuous weight records of 16 men (average weight of 157 lb. in December, 1947) and recorded an average gain of 3.4 lb. per man from December 4, 1947 to April 22, 1948.

Caloric intake in relation to weather

A mass of observations during World War I and World War II have led to the generalization by Johnson and Kark ('47) that the colder the weather the more North American troops want to eat. The results of the present surveys are in agreement with this generalization.

During the three surveys temperatures ranged from +31° F. to —37° F., windchill from 400 to 2200, wind velocity from

TABLE 2

Nutrients in food consumed by troops at Fort Churchill, winter 1947–1948

NUTRIENT (Average per man per day)	PERIOD 9–18 Nov. 1947	15–24 Feb. 1948	11–20 Apr. 1948
Calories (cal.)	5,290	5,650	5,250
Protein (gm)	165	166	173
Fat (gm)	221	261	250
Carbohydrate (gm)	677	677	591
Calcium (mg)	1,455	1,636	1,159
Phosphorus (mg)	2,736	2,813	2,687
Iron (mg)	33	30	33
Vitamin A (I.U.)	20,278	10,543	13,010
Thiamine [1] (mg)	2.50	2.30	2.66
Riboflavin [1] (mg)	3.45	3.38	2.95
Niacin [1] (mg)	28.8	20.4	31.5
Ascorbic acid [1] (mg)	139	100	124
Average number of men fed daily	95	111	77

[1] Corrected for assumed cooking losses.

6 m.p.h. to 50 m.p.h., daily precipitation from 0.00 inches to 0.14 inches of snow (water equivalent), and relative humidity from 70% to 100%. Figure 1 shows the total caloric intake (mess intake plus canteen purchases) in relation to weather conditions prevailing at the time of the surveys. The caloric intake was inversely correlated with the mean environmental temperatures prevailing at the times of the three surveys, and was directly correlated with the mean windchill. In other

words, the troops voluntarily regulated their food intake in relation to the severity of the outdoor weather.

Pattern of food intake

Another important generalization by Johnson and Kark ('47) which emerged from observations in World War I and

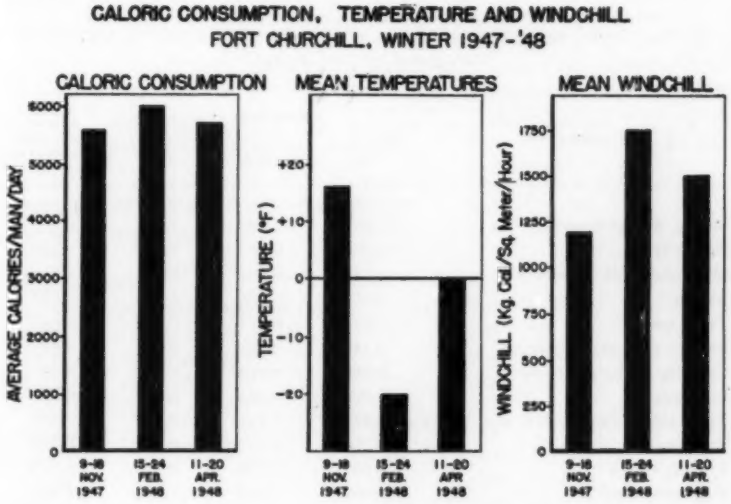

Fig. 1 Total caloric intake of troops at Fort Churchill in winter of 1947–1948 compared with mean windchill and mean temperatures prevailing at times of nutritional surveys. Ordinates, from left to right: Total average caloric intake daily per man. Mean temperature, expressed as degrees Fahrenheit. Windchill, expressed as kilogram calories per square meter per hour. Abscissae represent 10-day periods of each survey.

World War II was that in all climates the relative proportions of protein, fat, carbohydrate and vitamins consumed were suprisingly constant. The present surveys add further support to this generalization, particularly in relation to protein intake and actual consumption of food items.

Consumption of food items by groups is shown in table 3 for the present surveys and for United States soldiers in the

Zone of the Interior as reported by Howe and Berryman ('45) for 1943. There is only one group of foods in which the arctic ration was consistently lower than the United States garrison ration — milk and milk products. This situation was caused by a supply problem, the inability to secure fresh milk. Where the consumption of lard and other fats was low in the arctic

TABLE 3

Average food consumption of soldiers at Fort Churchill, winter of 1947–1948, calculated by food groups

FOOD GROUPS	FORT CHURCHILL			U. S. ARMY [1] CAMPS 1941–1943 (AVERAGE OF 455 SURVEYS)
	9–18 Nov. 1947	15–24 Feb. 1948	11–20 Apr. 1948	
	lb./man/day	lb./man/day	lb./man/day	lb./man/day
Meats, fish and poultry	1.023	0.980	1.272	0.909
Eggs, fresh	0.252	0.261	0.275	0.149
Milk and milk products	0.719	0.984	0.453	1.016
Butter	0.125	0.178	0.135	0.083
Fats, other	0.036	0.074	0.055	0.069
Sugars and carbohydrates	0.446	0.540	0.313	0.262
Cereals, grain products	0.966	0.922	0.911	0.567
Legumes	0.028	0.034	0.040	0.055
Vegetables, leafy green and yellow	0.846	0.248	0.430	0.359
Tomatoes	0.306	0.199	0.300	0.155
Fruits, citrus	0.317	0.179	0.222	0.248
Potatoes, white	0.990	0.948	1.197	0.561
Vegetables, other	0.511	0.465	0.508	0.252
Fruits, other	0.452	0.223	0.387	0.358
Cheese, Cheddar type	0.011	0.008	0.024	....
Fruits, dried	0	0.027	0	0.026
Waste (kitchen and plate)	0.75	0.51	0.38	0.32

[1] Data of Howe and Berryman ('45) for U. S. Army camps.

diet, it was offset by a higher intake of butter. Plate and kitchen waste were very high at first (0.75 lb./man/day) in comparison with U. S. Army camps listed in table 3 but after this situation was appreciated by the proper authorities steps were taken to reduce this figure, and by the end of the winter waste was down to a reasonable amount (0.38 lb./man/day).

Table 4 presents the average pattern of food consumption calculated by food groups and expressed as a percentage of the total food consumed. This type of calculation enables us to make a direct comparison between the patterns in U. S. Army training camps in temperate environments and those

TABLE 4

Average patterns of food consumption of soldiers at Fort Churchill, winter of 1947–1948, calculated by food groups and expressed as percentage of total consumption [1]

FOOD GROUPS	FORT CHURCHILL			U. S. ARMY CAMPS [2] 1941–1943 (AVERAGE OF 455 SURVEYS)
	9–18 Nov. 1948	15–24 Feb. 1948	11–20 Apr. 1948	
	%	%	%	%
Milk and milk products	10.1	15.7	7.0	20.0
Meats, fish and poultry	14.6	15.6	19.5	17.9
Cereals	13.7	14.7	14.0	11.2
Potatoes, white	14.1	15.1	18.3	11.1
Vegetables, leafy green and yellow	12.0	4.0	6.6	7.1
Fruits, other than citrus	6.4	3.6	5.9	7.1
Sugars and carbohydrates	6.3	8.6	4.8	5.2
Vegetables, other	7.3	7.4	7.8	5.0
Fruits, citrus	4.5	2.9	3.4	4.9
Tomatoes	4.4	3.2	4.6	3.1
Eggs, fresh	3.6	4.2	4.2	2.9
Butter	1.8	2.8	2.1	1.6
Fats, other	0.5	1.2	0.8	1.4
Legumes	0.5	0.5	0.6	1.0
Fruits, dried	0	0.4	0	0.5
Cheese, Cheddar type	0.2	0.1	0.4	0.0

[1] The data were analyzed statistically and showed no significant difference between the distributions.

[2] Data of Howe and Berryman ('45) for U. S. Army camps.

prevailing at Fort Churchill. Four points may be seen in the table. First, consumption of milk and milk products was low at Fort Churchill because of the supply problem mentioned above. Second, cereals and white potatoes comprised a higher percentage of the diet at Fort Churchill. Third, the sum of butter and other fats was not greatly different in the U. S.

army camps and at Fort Churchill. Fourth, there is no evidence of an increase in the appetite for any particular type of food during the winter. A statistical analysis of the data reveals no significant differences.

TABLE 5

Percentage of caloric intake contributed by protein, fat, and carbohydrate in rations eaten by troops in various environments

ENVIRONMENT AND PLACE	PROPORTION OF TOTAL CALORIES PROVIDED BY:		
	Protein	Fat	Carbohydrate
	%	%	%
1. Arctic and subarctic areas			
Fort Churchill,[1] 9–18 November, 1947	13	37	50
Fort Churchill,[1] 15–24 February, 1948	12	41	47
Fort Churchill,[1] 11–20 April, 1948	13	42	45
Exercise "Musk Ox,"[2] February–May, 1946	13	42	45
2. Temperate areas			
U. S. Army Zone of Interior,[3] 1941–1943	13	43	44
U. S. Army Zone of Interior,[4] Mountain Troops, September, 1946	14	44	42
3. Tropical areas			
U. S. Army on Guadalcanal,[5] 1945	13	34	53
U. S. Army on Hawaii,[5] 1945	13	33	54
U. S. Army on Guam,[5] 1945	13	32	55
U. S. Army on Iwo Jima,[5] 1945	13	33	54
U. S. Army on Luzon,[5] 1945	12	34	54

[1] Data from present surveys.
[2] Data from Kark et al. ('48).
[3] Data from Howe and Berryman ('45).
[4] Data from Crowley, Johnson and Anderson ('47).
[5] Data from Kark et al. ('47).

Table 5 shows the per cent of Calories furnished by protein, fat, and carbohydrate in arctic, temperate and tropical diets. The close agreement between the different arctic and subarctic diets, and the close similarity among the arctic, subarctic and temperate diets, add further substantiation to the findings of Kark et al. ('48) from the Canadian Exercise "Musk Ox" that North American troops living in the winter in the arctic do not voluntarily consume a higher proportion of fat than

do troops in temperate climates. The small variation in the protein intake in the entire table is striking, and again corroborates a statement of Johnson and Kark ('47) that North American troops voluntarily consume about the same proportion of protein in the diet regardless of environment.

SUMMARY

A study was made of the voluntary food consumption of garrison troops at Fort Churchill, Manitoba, Canada, during 10-day periods in November, 1947, February, 1948, and April, 1948. Each of the three nutrition surveys was conducted on troops who ate together in the same mess and received an abundant ration of fresh and frozen foods, providing 5,500 Cal. per man per day (Canadian Army Arctic Ration Scale 7).

The average daily consumption per man, including canteen (post exchange) purchases, in the three surveys was 5,620, 5,590 and 5,690 Cal., respectively. The intake of all nutrients was well above the allowances for active men recommended by the U. S. National Research Council. Throughout the winter the troops were in good health and maintained their body weights.

The caloric intake in the three surveys was inversely correlated with the mean outdoor temperatures prevailing at the time of survey, and was directly correlated with the mean windchill.

In all three surveys the percentages of Calories furnished by protein, fat and carbohydrate remained almost constant, averaging 13, 41 and 46, respectively. These values are not significantly different from those reported many times for United States troops eating a garrison ration in temperate climates. As judged by the consumption of individual food items, the pattern of food habits was not different in the subarctic climate af Fort Churchill from that in U. S. Army training camps in temperate climates. In particular, there was no evidence of an increased appetite for fats in the subarctic winter.

ACKNOWLEDGMENTS

Many thanks are due Lt. Col. A. J. Tedlie, D. S. O., W.O.I., M. J. Fallon and Sgt. N. Nichol of the Canadian Army and Lt. Col. W. E. Downing of the United States Army for their wholehearted cooperation during the conduct of these surveys at Fort Churchill.

Grateful acknowledgment is made to Dr. R. E. Johnson for his many helpful suggestions and criticisms.

Thanks are also due to Miss Evelyn Marek, G. Weigend, J. H. Bell and R. W. Powell for technical assistance in the chemical analyses.

LITERATURE CITED

BERRYMAN, G. H., AND C. CHATFIELD 1943 Short method of calculating nutritive value of diets. J. Nutrition. *25:* 23.

BERRYMAN, G. H., AND P. E. HOWE 1944 Short method of calculating nutritive value of diets (concluded). Ibid., *27:* 231 (correction, *27:* 434).

CROWLEY, L. V., R. E. JOHNSON AND G. V. ANDERSON 1947 Medical nutritional aspects of troops allowed free choice of any quantity of any item in a packaged ration. Fed. Proc., *6:* 440.

HOWE, P. E., AND G. H. BERRYMAN 1945 Average food consumption in training camps of the United States Army. Am. J. Physiol., *144:* 588.

JOHNSON, R. E., AND R. M. KARK 1947 Environment and food intake in man. Science, *105:* 378.

KARK, R. M., H. F. AITON, E. D. PEASE, W. B. BEAN, C. R. HENDERSON, R. E. JOHNSON AND L. M. RICHARDSON 1947 Tropical deterioration and nutrition. Clinical and biochemical observations on troops. Medicine, *26:* 1.

KARK, R. M., R. R. M. CROOME, J. CAWTHORPE, D. M. BELL, A. BRYANS, R. J. MACBETH, R. E. JOHNSON, F. C. CONSOLAZIO, J. L. POULIN, F. H. L. TAYLOR AND R. C. COGSWELL 1948 Observations on a mobile arctic force. The health, physical fitness and nutrition of Exercise "Musk Ox," February–May 1945. J. Appl. Physiol., *1:* 73.

KARK, R. M., R. E. JOHNSON AND J. S. LEWIS 1945 Defects of pemmican as an emergency ration for infantry troops. War Med., *7:* 345.

NATIONAL RESEARCH COUNCIL 1945 Tables of food composition. Food and Nutrition Board, Committee on Food Composition, Washington, D. C.

———— 1948 Recommended dietary allowances. Food and Nutrition Board, Committee on Dietary Allowances, Washington, D. C.

SIPLE, P. F. 1945 Quoted in Clothing Test Methods, Pub. C. A. M. No. 390, U. S. National Research Council, Washington, D. C.

THE PRODUCTION OF BIOTIN DEFICIENCY IN THE MOUSE[1, 2]

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(Received for publication January 21, 1949)

TWO FIGURES

INTRODUCTION

There are relatively few papers in the literature dealing with biotin deficiency in the mouse. In a recent review of the vitamin requirements of the mouse, Morris ('47) cited in this connection only the work of Nielsen and Black ('44), who found that better growth was obtained in mice on a synthetic diet when biotin was added than when it was omitted. Complete symptoms of the deficiency were obtained only when 0.6% sulfasuxidine was added to the diet. Morris considered these experiments to be not very clear-cut because of the poor growth response of the mice to the basal ration, since other investigators have obtained good growth in mice on similar basal rations without the addition of biotin and folic acid.

Lichstein, Waisman, McCall, Elvehjem and Clark ('45), in experiments to test the influence of biotin on the susceptibility of Swiss mice to experimental poliomyelitis, produced the

[1] Part of the material in this paper is from a thesis presented by Diane H. Winston in partial fulfillment of the requirements for the Sc.M. degree, Brown University, 1948.

[2] Supported in part by grants from the National Advisory Cancer Council and the Rhode Island Cancer Society.

[3] Horton-Hallowell Fellow of Wellesley College, 1947–48. National Cancer Institute Postdoctorate Research Fellow, 1948–49.

deficiency by including 20% powdered egg albumin in a synthetic diet. They report symptoms of deficiency like those that occur in the rat, appearing after 4 to 8 weeks on the diet. They had difficulty with diarrhea which they attributed to *Salmonella* infection, and suggested that mice deficient in biotin are more susceptible to naturally occurring infections than are animals maintained on a diet optimum in biotin. Since the diarrhea was fatal before the 12th day, it appears that it may have been due to an effect on the intestinal flora that precedes depletion of the biotin in mice. No effect of the deficiency on susceptibility to poliomyelitis was observed.

Kligler, Guggenheim and Herrnheiser ('46) in their study of biotin deficiency and resistance to infection used a diet containing egg white and found an increased susceptibility of mice to *Salmonella* infection. Since their experiments did not run over 24 days and they report none of the usual symptoms of the deficiency, it may be questioned whether the effect they obtained was due to the production of biotin deficiency or was merely due to an effect on the intestinal flora which may be directly influenced by a diet poor in biotin.

In our own experiments we have adapted for mice the diets used by various investigators for rats, and have consistently obtained the deficiency.

EXPERIMENTAL

The mice used were of a strain of albinos that is being developed in this laboratory. It is inbred and selected for vigor of growth and reproductive performance. The mouse room is kept at a constant temperature of from 75° to 78° F. The stock diet is a commercial chow [4] supplemented by rolled oats for nursing mothers and young up to 4 weeks of age. In the dietary experiments mice are kept in glass cages with floors of wire mesh raised from the bottom to minimize coprophagy.

For production of the deficiency several diets have been used. These are presented in summary in table 1. In all cases dried egg white has been included to insure the unavailablity

[4] Purina Laboratory Chow.

of biotin, and yeast and cod liver oil have been used to supply the vitamins. We have used two types of egg white: a yellowish flake produced by drying fresh white of egg, and a powder produced by drying white of egg after fermentation to remove sugar. The latter corresponds to the imported "Chinese egg white" of earlier experimenters. It had been suggested to us that some of the avidin might be destroyed in the fermenting

TABLE 1

Composition of diets[1]

INGREDIENT	DIET NO.					
	1D	1E	2A	5B	5C	9A
Vitamin-test casein (GBI[2])	30	15	30	10	10	10
Egg white	..	15	..	20	20	20
Cornstarch (Argo)	..	..	29	29	14	28.5
Sucrose	47	47	..	..	15	..
Crisco	..	..	20	20	20	20
Corn oil (Mazola)	7	7	4	4	4	4
Cod liver oil (Squibb)	2	2	2	2	2	2
Powdered brewers' yeast (GBI)	8	8	8	8	8	8
Salt mixture USP XII, no. 2 (GBI)	4	4	7	7	7	7
Dried liver	2	2	..	..	..	..
Sulfaguanidine	..	..	..	..	..	0.5

[1] Several minor variants of these diets have been used in which Crisco replaced the corn oil, or sucrose all or a part of the cornstarch, or dried liver a part of the cornstarch, but no significant differences were observed.

We thank Henningsen Lamesa, Inc., for the egg white; Merck and Co., Inc., for the biotin; the Lederle Laboratory Div., American Cyanamid Co., for the sulfaguanidine; and Armour and Co. for the dried liver.

[2] General Biochemicals, Incorporated.

process, so that the use of the unfermented material might be desirable. We have made no quantitative studies but at the level we have been feeding egg white no significant difference between the two types is apparent. Inasmuch as the unfermented material must be refrigerated to avoid deterioration and must also be ground in a ball mill to assure its proper incorporation in the diet, we have found it more convenient to use the powdered fermented material.

In our first experiments a diet modified from that of Emerson and Keresztesy ('42) was employed. In all later experiments we have used a basal diet in general use in other investigations in this laboratory, differing primarily in that it contains a higher percentage of fat. This makes it easier to pack firmly in the food dishes and prevents to some extent the scattering of the food, although at best there is considerable wastage. It is also our feeling that the mice eat the diet with the higher fat content more eagerly and grow faster, which contributes to the early production of the symptoms. This is not in keeping with the observation of Nielsen and Elvehjem ('42) that fat seems to have a slight sparing action on biotin deficiency, particularly as far as the time of appearance of the paralysis is concerned. These workers fed to rats diets containing (a) no fat, (b) 5% corn oil, or (c) 41% lard with 10% egg white, and observed paralysis at 9, 9 to 12 and 12 to 14 weeks, respectively. Our rations contained 26% fat and 20% egg white, which may explain the difference in our experience.

We have used cornstarch rather than sugar as a carbohydrate in most experiments because there appeared to be no advantage in employing sugar in those experiments where it was used. Boas ('27) observed that potato starch gave some protection against egg white injury in rats, but that cornstarch had no such effect.

The dried liver was sometimes used during the first few days that the mice were on the diet to minimize the diarrhea which frequently occurs (Parsons, '31).

For cure of the deficiency symptoms we have injected biotin subcutaneously or pulped mouse liver intraperitoneally or have substituted the corresponding basal diet for the one containing egg white.

The biotin was given in a dose of either 2 μg or 4 μg a day. The liver was prepared by grinding the liver of one adult mouse in a glass tissue grinder with 2 ml of Ringer's fluid. A single injection of 0.5 ml of this mixture was the customary dose.

We have, in the last two years, conducted 20 different experiments using these various diets to produce biotin deficiency and have used over 150 mice. Since the purposes of the experiments have varied, and consequently the details, only two have been selected for detailed presentation.

Experiment 167. Production of biotin deficiency on diet containing egg white

Nov. 7: Two litters of 8 mice each aged 16 days, weights 5.2 to 7.4 gm, placed on diet no. 5B with 2% dried liver.
Nov. 9: Four developed diarrhea; three died and one recovered.
Nov. 24: Changed to diet 5B without the dried liver.
Nov. 29: (Twenty-two days on diet.) First symptoms in one animal, eyes swollen and much fur gone.
Dec. 5: Practically no gain in weight since Nov. 29.
Dec. 16: Symptoms generally developing.
Jan. 9: Two animals had developed no symptoms and had continued to gain weight. All the rest had severe symptoms, including kargaroo posture in several. Seven mice used in another experiment. Four with mild symptoms retained.
Jan. 22 to Feb. 19: The remaining animals, with severe symptoms, used in other experiments.

This experiment shows the variability in time of appearance and in severity of the symptoms, the early diarrhea, and the failure of two animals to develop any symptoms long after the rest had clearly done so. The average growth curve of these mice is shown in figure 1.

This figure shows the average growth curve of two mice on a control diet (2A) of the same composition except that the egg white was replaced by casein. Average growth curves of mice in two other experiments are also presented: Experiment 168 with 14 animals on the same diet used in experiment 167, and experiment 184 with mice on diet 5C, in which part of the cornstarch is replaced by sucrose.

Experiment 285. Production of biotin deficiency in mice on a diet containing egg white and 0.5% sulfaguanidine

July 31: Four mice from one litter, aged 18 days, weights 9 to 10.7 gm, placed on diet no. 9A. No record of diarrhea.
Aug. 19: Excellent gain in weight; weights now 21.7 to 26.6 gm.

Sept. 2: Almost no additional gain in weight, animals all show loss of hair and swollen red skin around lower lip (33 days on diet).

Sept. 20: Animals now losing weight; symptoms severe; 51 days on diet. Daily injections of 2 μg of biotin begun for three animals, the 4th being given none. All were retained on the diet.

Sept. 27: Slight improvement — dose of biotin increased to 4 μg.

Sept. 29: All treated animals gaining weight and eyes and skin improved.

Oct. 1–4: Hair generally returning on all bare spots.

Oct. 11: Untreated animal with extreme symptoms sacrificed for histological study.

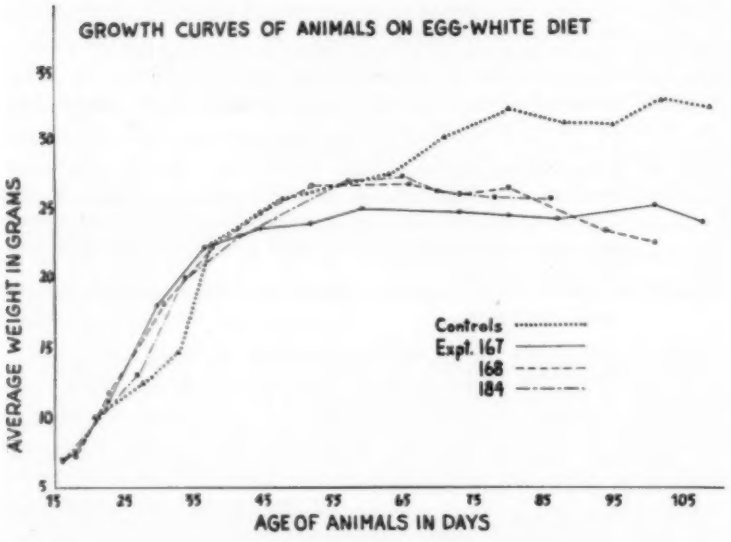

Figure 1

Oct. 22: Treated mice look perfectly normal; female put with males.

Oct. 24: Female has vaginal plug.

Nov. 11: Female gave birth to 4 young which she failed to nurse.

Nov. 13: One male sacrificed for histological study.

Dec. 3: Female and male still thriving; sacrificed for study of intestinal flora and histology.

The individual growth curves of these mice are shown in figure 2. The occurrence of the symptoms was strikingly uniform. Histological study showed that there was no goitrogenic effect of the sulfaguanidine. Complete recovery result-

ing from injection of biotin while still on the diet indicates that there is no toxic effect of the drug and that the deficiency is a simple one.

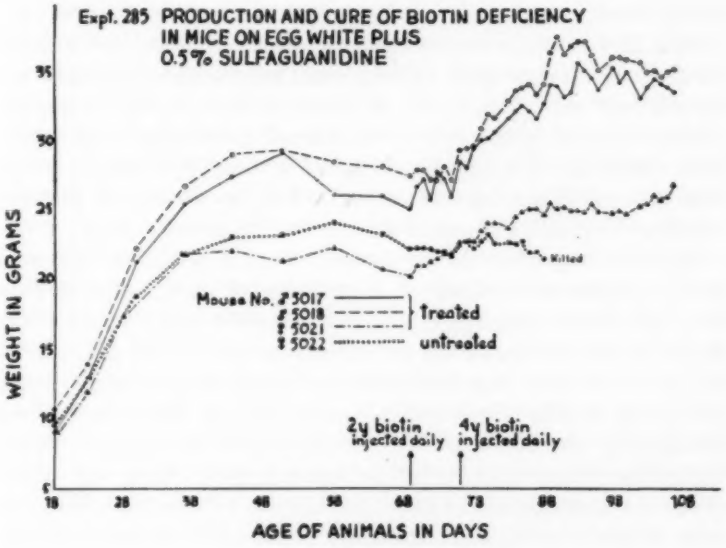

Figure 2

DISCUSSION

We have obtained biotin deficient mice by feeding all of the diets in table 1 that contain egg white. At one time or another we have seen in the mouse most of the symptoms and phenomena described by others as occurring in the rat, and in all cases where a cure has been attempted it has been effected. In our more recent experiments with diet 5B using 16-day-old mice, recognizable symptoms begin to appear in three weeks and by 4 or 5 weeks the symptoms are extreme. The animals seem to grow well until about the time the symptoms begin to appear, when the growth curve falls off to a plateau (fig. 1). The excellent description by Sullivan and Nicholls ('42) and Sullivan, Kolb and Nicholls ('42) of the appearance

of the symptoms in the rat and their disappearance as curative treatment becomes effective is generally applicable to the mouse, with the single exception that the considerable accumulation of brownish seborrheal material on the skin which occurs in the rat seems to be absent in the mouse.

In a few animals the symptoms fail to appear. An animal without symptoms may be in a cage where the rest have severe symptoms. This seems in many cases to be due to coprophagy in spite of the raised wire mesh bottoms of the cages. The fact that the intestinal bacteria may synthesize large amounts of biotin has been suggested or shown by several workers (e.g., McGregor, Parsons and Peterson, '47).

In a few experiments we have used C_{57} black mice. Several investigators have reported a graying of black hairs in rats on a diet containing egg white, but Kennedy and Palmer ('45) failed to obtain it in their experiments. In the C_{57} black mice we observed that the hair becomes rusty and in some cases gray. As in the white mice, some areas of the skin become completely denuded. After the injection of 2 μg of biotin daily for a few days, with the animals still on an egg white diet, new hair begins to grow out on the bare areas. While it may be gray at first, it ultimately becomes black and shining. Emerson and Keresztesy ('42), on the other hand, reported that rats rendered biotin deficient on a diet containing egg white showed a symmetrical graying of the ingrowing fur after treatment with biotin for 12 days and that, furthermore, the gray might persist through 5 weeks of treatment. They themselves point out, however, that the persistence of the "spectacle eye" condition may indicate that their dose was suboptimum. We would tentatively conclude, therefore, that biotin is directly or indirectly a necessary factor in the development of black hair color.

From reports in the literature it would appear that when the rat is used in the study of biotin deficiency, the production of the deficiency is more consistent than we have found it in the mouse. One source of variation in results may be the initial state of the experimental animals. Sullivan and Nicholls

('42) have summarized the evidence in the literature in favor of using very young animals, preferably from mothers on diets poor in biotin. Our best results have been obtained with young weaned as early as possible on the egg white diet, preferably on the 16th to the 18th day of age. The nature of the intestinal flora must also be a cause of variation, since it is normally a source of considerable biotin. Gall, Fenton and Cowgill ('47) have shown that the cecal flora of different strains of mice may be different. This may be the explanation of the unexpected behavior of the mice in the experiments of Nielsen and Black ('44), which on a biotin low basal diet that would ordinarily produce no deficiency showed marked increase in growth when biotin was added. They concluded that the mouse needs a dietary source of biotin. Fenton, Cowgill and Stone ('48), however, report that with their purified diets and under the conditions of their experiments they have not found that the mouse requires a dietary source of biotin or folic acid. To explain the discrepancy they suggest the possibility that the requirement for biotin and folic acid may be different in different strains. That the difference in requirement may be due to differences in cecal flora is an obvious possibility. The constancy of our results when the change in intestinal flora was controlled by the use of sulfa drugs, which is discussed below, is evidence of the importance of the intestinal flora as a source of variation.

A disturbing effect sometimes produced when the young mice are first placed on the egg white diet is a diarrhea which in a few cases may be fatal. This has been observed in rats by many workers (Parsons, '31; Parsons and Kelly, '33; and Salmon and Goodman, '34). In agreement with Bateman ('16), we have found no significant intestinal inflammation accompanying the diarrhea. On the other hand, Lichstein, Waisman, McCall, Elvehjem and Clark ('45) report an acute inflammation of the intestinal tract and enlargement of the spleen. It is generally agreed that the diarrhea is not due to biotin deficiency but to some other action of the egg white diet. It seems possible that it may be due to a change in the

intestinal flora as a result of the lack of available biotin in the food. Lichstein and his co-workers found large numbers of organisms in the feces which they identified as probably *Salmonella typhimurium*, which may account for the difference in our findings; in our animals the infection may have been a less virulent one. This would also account for the fact that our animals frequently recover and thrive for some time. The diarrhea has not occurred when sulfaguanidine has been used with the egg white in the diet. This would suggest that the coliform organisms are responsible for the diarrhea when it does occur, for it is the coliform population in particular that is reduced by the sulfa drugs (Evenson, McCoy, Geyer and Elvehjem, '46).

Several investigators have shown that the inclusion of the less readily absorbed sulfa drugs, sulfaguanidine and sulfasuxidine, in a semisynthetic diet produces a complicated deficiency in rats that can be prevented or cured by combined treatment with folic acid and biotin. The consensus is that this is due to a change in the intestinal flora. Evenson et al. ('46) have shown that the coliform organisms are almost eliminated after 5 days on a diet containing 0.5% sulfasuxidine. Gant, Ransone, McCoy and Elvehjem ('43) conclude that it is the coliform organisms that produce the biotin and folic acid in the intestine, and that this source makes the production of the deficiency irregular with egg white and prevents it on a diet low in biotin. Nielsen, Shull and Peterson ('42) have shown that synthesis of biotin is almost arrested in the rat on a diet low in biotin with 0.5% sulfaguanidine. In our experiments the sulfaguanidine has been added to a diet containing egg white which also contains yeast. This diet has been the most successful in the production of the symptoms in the mouse. After symptoms were completely developed, they were rapidly cured by injection of two to 4 μg of biotin subcutaneously daily while the mice were still on the diet containing sulfaguanidine and egg white. One female mouse gave birth to a litter on this regimen. She had been on the diet

since the age of 18 days and had developed and recovered from a severe biotin deficiency.

The deficiency obtained with sulfa drugs when pure vitamin supplements without folic acid and biotin are used apparently involves deficiency not only in these two but also in pantothenic acid (Black, Overman, Elvehjem and Link, '42) and possibly vitamin K (Wright and Welch, '44). In our experiments, however, the deficiency is produced by supplying avidin in egg white which combines with the available biotin of the diet, and at the same time supplying sulfaguanidine which eliminates a large part of the biotin synthesis in the intestine. Since the symptoms are cured by the subcutaneous injection of biotin, the deficiency must be a simple one involving biotin alone. Emerson and Wurtz ('45) have used sulfasuxidine in conjunction with egg white to produce biotin deficiency in the rat. They also considered that a simple biotin deficiency was produced because the symptoms were prevented or overcome by the oral administration of 5 μg of biotin daily.

The possibility of toxicity of the sulfaguanidine must be considered. It has been shown to be goitrogenic in rats but at a higher level than we have fed it (Mackenzie, Mackenzie and McCollum, '41). Black, McKibbin and Elvehjem ('41), however, found no harmful effects in rats receiving 0.5% in the diet. We have found none in mice after many weeks with 0.5% in the diet. It would be surprising if any goitrogenic effect appeared at this level in the mouse, since even such a vigorous goitrogenic agent as thiouracil must be used in the mouse at a level much higher than in the rat to produce the effect. Other pathological effects following the administration of sulfaguanidine have been described by Daft, Ashburn, Spicer and Sebrell ('42), Daft, Ashburn and Sebrell ('42), and Spicer, Daft, Sebrell and Ashburn ('42). These involve changes in arterial walls, muscles, and liver. Welch and Wright ('43), however, found no such effects and were unable to explain the discrepancy. Although we have made no systematic study, we have seen no evidence of such changes.

The fact that the animals recover from severe symptoms while still on the diet containing egg white and sulfaguanidine when biotin is injected daily, and that they thrive and grow and are able to reproduce, would seem to indicate not only that the deficiency is a simple one, but that the sulfaguanidine is not toxic when fed to mice at the level used. This would, then, seem to be the best method of producing biotin deficiency in the mouse quickly and certainly.

SUMMARY AND CONCLUSIONS

1. Biotin deficiency has been produced in mice by diets containing dried egg white with and without the addition of sulfaguanidine.

2. All of the symptoms described in the rat have been observed in the mouse except the accumulation of brownish seborrheal material on the skin.

3. The appearance of the symptoms of the deficiency have been variable on the egg white diet without sulfaguanidine. The sources of this variability are discussed. With sulfaguanidine the results have been consistent.

4. The fur of C_{57} black mice becomes rusty or gray on the deficiency-producing diet. It becomes black when biotin is injected while the animals are still on the same diet. It is concluded that biotin is directly or indirectly a necessary factor in the development of black hair color.

5. The diarrhea which may occur soon after mice are placed on the egg white diet is attributed to a change in the intestinal flora.

6. Sulfaguanidine at the level used, 0.5% in the diet, is not toxic.

7. The deficiency produced by the combination of egg white and sulfaguanidine is a simple one since it can be cured by the injection of biotin while the animals are still on the diet.

LITERATURE CITED

BATEMAN, W. G. 1916 The digestibility and utilization of egg protein. J. Biol. Chem., *26:* 263.

BLACK, S., J. M. MCKIBBIN AND C. A. ELVEHJEM 1941 Use of sulfaguanidine in nutrition experiments. Proc. Soc. Exp. Biol. Med., *47:* 308.

BLACK, S., R. S. OVERMAN, C. A. ELVEHJEM AND K. P. LINK 1942 The effect of sulfaguanidine on rat growth and plasma prothrombin. J. Biol. Chem., *145:* 137.

BOAS, M. A. 1927 The effect of desiccation upon the nutritive properties of egg-white. Biochem, J., *21:* 712.

DAFT, F. S., L. L. ASHBURN AND W. H. SEBRELL 1942 Biotin deficiency and other changes in rats given sulfanilylguanidine or succinylsulfathiazole in purified diets. Science, *96:* 321.

DAFT, F. S., L. L. ASHBURN, S. S. SPICER AND W. H. SEBRELL 1942 The occurrence of hyaline sclerosis and calcification of blood vessels in rats on sulfaguanidine. Pub. Health Rep., *57:* 217.

EMERSON, G. A., AND J. C. KERESZTESY 1942 Biotin deficiency in the rat. Proc. Soc. Exp. Biol. Med., *51:* 358.

EMERSON, G. A., AND E. WURTZ 1945 Failure of succinylsulfathiazole to aggravate egg white induced biotin deficiency in rats. Ibid., *59:* 297.

EVERSON, A., E. MCCOY, B. R. GEYER AND C. A. ELVEHJEM 1946 The cecal flora of white rats on a purified diet and its modification by succinylsulfathiazole. J. Bact., *51:* 513.

FENTON, P. F., G. R. COWGILL AND M. A. STONE 1948 Long-term maintenance of two strains on synthetic and on stock diets. Proc. Soc. Exp. Biol. Med., *67:* 27.

GALL, L. S., P. F. FENTON AND G. R. COWGILL 1947 Nutrition of the mouse. IV. Comparison of bacterial population of two highly inbred strains. Ibid., *66:* 414.

GANT, O. K., B. RANSONE, E. MCCOY AND C. A. ELVEHJEM 1943 Intestinal flora of rats on purified diets containing sulfonamides. Ibid., *52:* 276.

KENNEDY, C., AND L. S. PALMER 1945 Biotin deficiency in relation to reproduction and lactation. Arch. Biochem., *7:* 9.

KLIGLER, I. J., K. GUGGENHEIM AND H. HERRNHEISER 1946 Nutritional deficiency and resistance to infection. The effect of biotin deficiency on the susceptibility of rats and mice to infection with *Salmonella typhimurium*. J. Inf. Dis., *78:* 60.

LICHSTEIN, H. C., H. A. WAISMAN, K. B. MCCALL, C. A. ELVEHJEM AND P. F. CLARK 1945 Influence of pyridoxine, inositol and biotin on susceptibility of Swiss mice to experimental poliomyelitis. Proc. Soc. Exp. Biol. Med., *60:* 279.

MACKENZIE, J. B., C. G. MACKENZIE AND E. V. MCCOLLUM 1941 The effect of sulfanilylguanidine on the thyroid of the rat. Science, *94:* 518.

MCGREGOR, M. A., H. T. PARSONS AND W. H. PETERSON 1947 Biotin balance in the albino rat. J. Nutrition, *33:* 517.

MORRIS, H. P. 1947 Vitamin requirements of the mouse. Vitamins and Hormones, *5:* 175.

NIELSEN, E., AND ... BLACK 1944 Biotin and folic acid deficiencies in the mouse. J. Nutrition, *28:* 203.

NIELSEN, E., AND C. A. ELVEHJEM 1942 Cure of paralysis in rats with biotin concentrates and crystalline biotin. J. Biol. Chem., *144:* 405.

NIELSEN, E., G. M. SHULL AND W. H. PETERSON 1942 Response of bacteria, yeast, and rats to peroxide-treated biotin. Intestinal synthesis of biotin in the rat. J. Nutrition, *24:* 523.

PARSONS, H. T. 1931 The physiological effects of diets rich in egg white. J. Biol. Chem., *90:* 351.

PARSONS, H. T., AND E. KELLY 1933 The effect of heating egg white on certain characteristic pellagra-like manifestations produced in rats by its dietary use. Am. J. Physiol., *104:* 150.

SALMON, W. D., AND J. G. GOODMAN 1934 Studies of the raw egg white syndrome in rats. J. Nutrition, *8:* 1.

SPICER, S. S., F. S. DAFT, W. H. SEBRELL AND L. L. ASHBURN 1942 Prevention and treatment of agranulocytosis and leukopenia in rats given sulfanilylguanidine or succinylsulfathiazole in purified diets. Pub. Health Rep., *57:* 1559.

SULLIVAN, M., L. KOLB AND J. NICHOLLS 1942 Nutritional dermatoses in the rat. VII. Notes on the posture, gait, and hypertonicity resulting from a diet containing unheated, dried egg white as the source of protein. Bull. Johns Hopkins Hosp., *70:* 177.

SULLIVAN, M., AND J. NICHOLLS 1942 Nutritional dermatoses in the rat. V. Signs and symptoms resulting from a diet containing unheated dried egg white as the source of protein. Arch. Derm. Syph., Chicago, *45:* 295.

WELCH, A. D., AND L. D. WRIGHT 1943 The role of "folic acid" and biotin in the nutrition of the rat. J. Nutrition, *25:* 555.

WRIGHT, L. D., AND A. D. WELCH 1944 Folic acid, biotin, and pantothenic acid deficiency and the liver storage of various vitamins in rats fed succinylsulfathiazole in highly purified rations. Ibid., *27:* 55.

THE WRIST STIFFNESS SYNDROME IN GUINEA PIGS

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(Received for publication January 11, 1949)

In 1936 Bahrs and Wulzen reported a wrist stiffness in guinea pigs which they considered to result from the lack of an antistiffness factor in the diet. Since then this deficiency syndrome has been studied in detail by van Wagtendonk and Wulzen and co-workers ('43–'47) at Oregon State College and, more recently, by Oleson et al. ('47) and Petering et al. ('48).

The Oregon group has described an impressive list of symptoms which characterize this deficiency disease, among which are a stiffness of the wrists and elbow joints, extensive calcification of the soft tissues, alterations of the concentrations of various phosphorus and calcium compounds in tissues, a decrease in the serum alkaline phosphatase activity, and others.

Among the crude dietary supplements studied, raw sugar cane juice has been reported to be the richest source of a factor which prevents or cures this joint stiffness. A white crystalline compound was isolated from cane juice by van Wagtendonk and Wulzen ('46) which reportedly cured wrist stiffness when fed in 5 daily doses of 0.002 μg each. In a more recent publication, Simonsen and van Wagtendonk ('47) reported that the activity of this crystalline material is actually due to traces of an impurity which, as pointed out by an anonymous writer in Nutrition Reviews ('48), means a factor active at

a level of the "order of 0.00002 microgram — a vanishingly small figure." Oleson et al. ('47), after finding that dried penicillin mold pad was curative and that this activity was found in the sterol fraction, tested 59 pure steroids and found that over 24 possessed some antistiffness activity. Of these compounds the esters of ergostanol were the most active. Petering et al. ('48) have reported confirmation of the Oregon work in part, i.e., the wrist stiffness. Ergostanyl acetate was found by these workers not to be as active as Oleson et al. had reported.

Two laboratories have published results which fail to confirm the Oregon work. Kon et al. ('46) and Homberger and Reed ('45) failed to demonstrate wrist stiffness in guinea pigs fed a skim milk diet (Wulzen and Bahrs, '41). Furthermore, these workers reported poor conditions of their animals and a high mortality, observations confirmed by Petering and colleagues ('48), Oleson and associates ('47), and Brown ('47). This high mortality on a skim milk diet led Petering and Oleson and their co-workers to use a different basal diet.

The present paper reports the results of our studies of this interesting but confusing syndrome.

MATERIALS AND METHODS

Two experiments are here reported involving over 200 guinea pigs. These guinea pigs, of mixed sexes and weighing 275–550 gm each, were obtained from a large local colony. They were divided at random into treatment groups and fed thereafter in individual wire screened cages. Five different diets were fed. Three of these diets were the same, insofar as was possible, as those described by the Oregon workers. Of these three, one was a *skim milk* diet (Wulzen and Bahrs, '41) supplemented with iron, copper, unsweetened orange juice, carotene, autoclaved oat straw and salt. Another was a *dry skim milk* diet supplemented with iron, copper, 14 vitamins and autoclaved straw. The *purified* diet (van Wagtendonk, Schocken and Wulzen, '44) included "vitamin-free" casein, cornstarch, irradiated yeast, salt mixture, orange juice,

mixed tocopherols and autoclaved straw. The irradiated yeast contained 9000 I.U. of vitamin D per gram and at the level fed (10%) furnished a large excess of this vitamin. The 4th diet, designated as the *Lederle diet,* consisted of natural feedstuffs and was reported by Oleson et al. ('47) to produce a stiffness of the wrists. The 5th diet was a commercial *calf meal* which is widely used as a rat stock diet (Maynard, '30), supplemented with salt, autoclaved straw and ascorbic acid (7 mg per day). The phosphorus content of the calf meal was increased by adding 2% of NaH_2PO_4. The calcium and phosphorous contents of the purified, Lederle and calf meal diets were, respectively, 0.61% and 0.73%; 0.59% and 0.79%; and 0.59% and 1.25%. The stock diet fed to some animals consisted of whole oats, mixed legume hay, fresh forage and iodized salt. Water was available to all animals. More than usual care was taken in shifting the guinea pigs from a stock diet to the experimental diets. In most cases a period of three weeks was taken for a complete change of diets, including the force feeding of those animals that were reluctant to change.

The supplements fed as sources of the "antistiffness factor" included crude sugar cane juice,[1] sugar cane bagasse,[2] and dried penicillin mold pad.[3] The cane juice was fed at a level of 0.25 ml per pig per day; the bagasse ad libitum (about 15 gm per day); and the penicillin mold pad incorporated in the diet at the level of 5%.

The wrists were tested for stiffness according to the technique described by van Wagtendonk and Wulzen ('46). All testing was done by one of the present authors (S.E.S.) and every attempt was made to make the observations as objective as possible. It soon became apparent to us that the subjectiveness of this method, along with the variability encountered within guinea pigs, did not justify the relatively precise scale of 1–4 used by other workers. Accordingly, a more conservative scale of judging the degree of stiffness was used, as

[1] Courtesy of Dr. A. G. Keller, Louisiana State University.

[2] See footnote 1.

[3] Courtesy of Dr. J. J. Oleson, Lederle Laboratories.

follows: 0 indicates normal flexing of the wrists; 1 signifies definite stiffness, and 2 denotes a wrist joint that is practically immobile.

Numerous X-ray pictures were taken of stiff and normal wrist joints.

RESULTS

The treatments used and some of the results are summarized in table 1.

In the first experiment routine testing of the wrists was started, but the high rate of mortality indicated the unsatisfactory nature of the Oregon diets and the testing was abandoned. Mortality was particularly high (96%) in those animals fed the skim milk diet with or without the supplement

TABLE 1

Summary of treatments and results

DIET	GUINEA PIGS					MORTALITY
	No. used	Av. initial wt.	No. stiff			
			Consistent	At times	Negative	
		gm				
First experiment						
Skim milk	23	335				22 in 2–3 wks.
Skim milk + cane juice	15	321				15 in 2–3 wks.
Purified	15	360				10 in 2–25 wks.
Purified + cane juice	15	332				10 in 2–28 wks.
Dry skim milk	15	302				10 in 3–7 wks.
Dry skim milk + cane juice	15	286				10 in 1–12 wks.
Stock	22	292				2 in 4 wks.
Second experiment						
Lederle	15	460	6	7	2	0 in 36 wks.
Lederle + mold pad	15	473	10	3	2	5 in 11–36 wks.
Purified	17	410	2	4	11	15 in 2–18 wks.
Purified + mold pad	8	452	0	1	7	8 in 2–17 wks.
Purified + bagasse	9	454	1	1	7	6 in 2–20 wks.
Calf meal	5	519	3	1	1	2 in 15 wks.
Calf meal + mold pad	5	523	1	2	2	1 in 3 wks.
Calf meal + bagasse	5	475	2	1	2	2 in 3–7 wks.
Dry skim milk	12	500	0	3	9	12 in 2–10 wks.
Dry skim milk + cane juice	5	473	2	0	3	4 in 3–4 wks.

of cane juice. Those guinea pigs fed the dry skim milk diet with or without cane juice fared little better (66% mortality). Although mortality was also high in those fed the purified diet, the guinea pigs lived for a longer period of time.

In the meantime our attention had been directed to a diet of natural feedstuffs used successfully to develop a wrist stiffness in the Lederle Laboratories and plans were made to study it, as well as a commercial calf meal enriched in phosphorus which in the past had been shown in this laboratory to produce extensive soft tissue calcification in the rat. Also, additional groups were fed the purified and the dry skim milk diets used by the Oregon workers supplemented with other reported sources of the "antistiffness factor."

In the second experiment, summarized in table 1, it will be noted that those animals fed the dry skim milk diet again sufferred a high mortality (100%), even when the ration was supplemented with cane juice. Mortality in those fed the purified diet was also high even when supplements of penicillin mold pad or sugar cane bagasse were fed. On the contrary, mortality was low in those animals fed either the Lederle or the calf meal diets.

In the second experiment the wrists of all animals were routinely tested over a period of 40 weeks. In agreement with the experiences of others, some guinea pigs (19%) had stiff wrists when received from the dealer. It is noted in table 1 that a high percentage of those fed the Lederle diet and the calf meal developed stiff wrists. The table designates the number which were consistently stiff over a period of 40 weeks, as well as those which varied from time to time. This variation in our animals was large even with the conservative scale which we used. This observation is in disagreement with Petering et al. ('48), who stated that "no spontaneous cures were observed in our animals," but is in agreement with Oleson et al., who observed about 13% of spontaneous cures. As an example of the variation which we encountered, table 2 presents periodic observations of the stiffness of the wrists of the 15 guinea pigs fed the Lederle basal diet. Our data give no

evidence that either penicillin mold pad or sugar cane bagasse significantly affects the incidence of wrist stiffness. Some stiff wrists were observed in the guinea pigs fed the purified diet, but the incidence was not nearly so high as in those fed either the Lederle or the calf meal diets. The guinea pigs fed the dry skim milk diet did not survive long enough to permit a valid test of their wrists.

TABLE 2

Variability of wrist stiffness in guinea pigs over a period of 40 weeks

GUINEA PIG [1] NO.	VARIABILITY														
1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0
2	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
4	0	0	1	1	1	0	1	1	1	1	1	1	0	0	0
5	0	1	2	2	1	1	2	2	2	1	1	1	0	1	1
6	2	1	1	1	2	1	2	2	2	2	2	2	1	2	2
7	0	0	0	0	1	0	1	1	2	0	0	0	0	1	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	1	1	1	1	1	1	2	1	1	1	1	2	2	2	2
10	1	2	1	2	1	2	1	1	1	2	2	2	1	1	2
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
13	1	1	1	1	1	0	0	1	1	1	0	1	0	0	0
14	0	0	0	1	1	1	0	1	0	1	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0

[1] Right wrist only.

Toward the end of the experiment 6 pairs of guinea pigs were carefully chosen to test the curative effects of ergostanyl acetate.[4] These animals were paired on the basis of previous treatment and degree of stiffness. They all were judged definitely stiff. One member of each pair was then fed 25 μg daily of ergostanyl acetate, 5 times the effective dose reported by Oleson et al. Wrists were tested at 5-day intervals. No difference was observed between the two groups either during the feeding of this supplement or at the end of 4 weeks. Petering et al. ('48) also found ergostanyl acetate ineffective at the

[4] Furnished by the Lederle Laboratories.

25 μg per day level, but did report a favorable response at a level of 100 μg per day.

X-ray pictures of the wrist joints of 16 stiff guinea pigs were compared with X-ray pictures of normal wrists in those guinea pigs fed the same diets, as well as with stock animals, and no evidence of any abnormality was noted.

The rates of growth are of secondary importance in this study and detailed data concerning them are not presented. The guinea pigs fed the skim milk or the dry skim milk diets, with or without cane juice, for the most part lost weight steadily from the beginning. Guinea pigs fed the purified diet with or without the various supplements did not grow but practically maintained their original weight until a short time before death. Those fed the Lederle or the calf meal diets made good weight gains throughout the experiment. The addition of the mold pad appeared to decrease food consumption and guinea pigs fed such rations made slower rates of gain than did other comparable animals.

It was previously mentioned that one of the symptoms described by the Oregon group was a calcification of the soft tissues. We have likewise observed this lesion, particularly in those fed the purified diet for a period of months. In these guinea pigs there appeared to be no correlation between the degree of tissue calcification and the wrist stiffness. Petering et al. ('48), using a modification of the Oregon purified diet (no irradiated yeast or cod liver oil), did not observe tissue calcification up to 4 to 6 months, even though the guinea pigs were very stiff. There is thus a suggestion that the wrist stiffness and the tissue calcification are different syndromes. This phase of the problem is being studied further and will be reported later.

DISCUSSION

To present a reasonable interpretation of the present position of the wrist stiffness syndrome in guinea pigs is difficult. It appears to be established, however, that guinea pigs will develop a stiffness of this joint when fed a rather wide variety of diets. In this aspect of the problem the Oregon workers

have been supported by the studies here described, although it is still difficult to see how this group could have published so many apparently clear-cut results when using diets that have in other laboratories led to such large weight losses and high rates of mortality. It is, of course, possible that there is a marked genetic difference in the animals used by the Oregon group and those used by the rest of us, but it is not probable that this would account for differences of such magnitude in the results obtained.

Confusion as to the identity of the "antistiffness factor" is even greater. A recent paper of Simonsen and van Wagtendonk ('47) indicating a protective factor effective at a level in the order of 0.00002 μg per day will not be accepted by many without more rigid proof. While some of the steroids studied by Oleson et al. ('47) may have antistiffness activity, the results reported here as well as those obtained by Petering et al. ('48) indicate a much lower potency than that published by Oleson and associates for the most active steroid — ergostanyl acetate. Petering et al. reported a favorable effect when 100 μg daily doses of ergostanyl acetate were fed. This interpretation was based on a change in average stiffness rating from 3.0 to 3.7 in 15 days, which was considered significant. A mean difference of 0.7 unit is indeed a small change to be considered significant, even when that change is objectively measured, as the wrist stiffness was not. The most recent paper from the Oregon workers (Christensen et al., '48) concluded that "the (wrist stiffness) assay procedure is not suitable for quantitative analytical purposes." In these published data mean differences within a treatment group differed as widely as 1.8 stiffness units. Evidence that sugar cane juice or bagasse or penicillin mold pad are rich in the antistiffness factor could not be confirmed in our studies.

The Oregon publications discuss the wrist stiffness and the tissue calcifications as if they were manifestations of the same deficiency syndrome. The preliminary observations reported here and the observations of Petering et al. suggest that they may be two different syndromes. The latter workers did not

observe calcification at all, even though severe stiffness was noted. Their basal diet differed from the Oregon purified diet in not containing irradiated yeast or cod liver oil. Whether or not the high level of vitamin D furnished by a diet containing 10% of irradiated yeast will lead to toxic calcification in the guinea pig is unknown. However, this is a possibility, since rough estimates show that such guinea pigs received approximately 22,000 I.U. of vitamin D for each 25 gm of diet consumed.

SUMMARY

The occurrence of stiff wrist joints and tissue calcifications in guinea pigs fed certain diets has been confirmed. In our hands the diets originally used by the Oregon workers have proved unsatisfactory for this purpose, and our evidence does not support the view that the joint stiffness and the tissue calcifications are a part of the same syndrome. Evidence was not obtained that sugar cane juice, sugar cane bagasse, penicillin mold pad or ergostanyl acetate possess antistiffness activity.

ACKNOWLEDGMENT

The authors are grateful to Dr. L. L. Barnes for taking and studying the X-ray pictures.

LITERATURE CITED

BAHRS, A. M., AND R. WULZEN 1936 A common factor in Planarian and mammalian nutrition. Physiol. Zool., *9:* 508.

BROWN, R. A. 1947 Personal communication.

CHRISTENSEN, B. E., M. B. NAPP, J. H. CHELDELIN AND R. WULZEN 1948 Studies on the guinea pig factor of Wulzen and van Wagtendonk. J. Biol. Chem., *175:* 275.

HOMBURGER, E., AND C. I. REED 1945 An effort to demonstrate wrist stiffness in guinea pigs on a deficient diet. Fed. Proc., *4:* 34.

KON, S. K., M. J. BIRD, M. E. COATES, J. E. C. MULLEN AND E. E. SHEPHEARD 1946 Failure to demonstrate wrist stiffness in guinea pigs receiving skim milk diets. Arch. Biochem., *11:* 371–373.

MAYNARD, L. A. 1930 A diet for stock rats. Science, *71:* 192.

NUTRITION REVIEWS 1948 Synthetic sterols and the guinea pig antistiffness factor. *6:* 106.

OLESON, J. J., E. C. VAN DONK, S. BERNSTEIN, L. DORFMAN AND Y. SUBBAROW 1947 Steroids and the stiffness syndrome in guinea pigs. J. Biol. Chem., *171:* 1–7.

PETERING, H. G., L. STUBBERFIELD AND R. A. DELOR 1948 A study of the assay method for the guinea pig antistiffness factor. Arch. Biochem., *18:* 487.

SIMONSEN, D. H., AND W. J. VAN WAGTENDONK 1947 A dietary factor essential for guinea pigs. XII. The *in vitro* influence of the antistiffness factor upon metabolism of liver tissue. J. Biol. Chem., *170:* 239.

VAN WAGTENDONK, W. J., AND R. WULZEN 1943 A dietary factor essential for guinea pigs. I. Isolation from raw cream. Arch. Biochem., *1:* 373–377.

VAN WAGTENDONK, W. J., V. SCHOCKEN AND R. WULZEN 1944 A dietary factor essential for guinea pigs. II. A comparative study of the creatine excretion of animals on a diet deficient in this factor and in vitamin E. Ibid., *3:* 305–310.

VAN WAGTENDONK, W. J. 1944 A dietary factor essential for guinea pigs. III. Changes in the distribution of acid-soluble phosphorus in the liver and kidneys during deficiency. J. Biol. Chem., *155:* 337–343.

VAN WAGTENDONK, W. J., A. S. RATHKEY, C. E. BALLOU AND R. WULZEN 1944 A dietary factor essential for guinea pigs. IV. Serum phosphatase during deficiency. Arch. Biochem., *5:* 273–278.

VAN WAGTENDONK, W. J., A. N. FREED AND C. E. BALLOU 1944 A dietary factor essential for guinea pigs. V. Phosphorus and calcium content of the blood and muscle during deficiency. Ibid., *5:* 329–335.

VAN WAGTENDONK, W. J., AND H. LAMFROM 1945 A dietary factor essential for guinea pigs. VI. Changes in the distribution of acid-soluble phosphorus in the muscle during a deficiency of the antistiffness factor. J. Biol. Chem., *158:* 421–424.

VAN WAGTENDONK, W. J., AND L. P. ZILL 1945 A dietary factor essential for guinea pigs. VII. Changes in the distribution of the plasma protein during the deficiency. Ibid., *159:* 247–253.

VAN WAGTENDONK, W. J., AND R. WULZEN 1946 A dietary factor essential for guinea pigs. VIII. The isolation of the antistiffness factor from cane juice. Ibid., *164:* 597–602.

VAN WAGTENDONK, W. J., AND D. H. SIMONSEN 1946 A dietary factor essential for guinea pigs. IX. Tissue metabolism during the deficiency. Ibid. *165:* 449–461.

VAN WAGTENDONK, W. J. 1947 A dietary factor essential for guinea pigs. X. Purine metabolism during deficiency. Ibid., *167:* 219–224.

VAN WAGTENDONK, W. J., AND A. M. FREED 1947 A dietary factor essential for guinea pigs. XI. Diffusible and non-diffusible plasma calcium during the deficiency of the antistiffness factor. Ibid., *167:* 225–228.

WULZEN, R., AND A. M. BAHRS 1941 Effects of milk diets on guinea pigs. Am. J. Physiol., *133:* 500.

OVULATION, FERTILIZATION, AND TRANSPORT OF OVA IN OLD, VITAMIN E DEFICIENT RATS[1]

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(Received for publication January 11, 1949)

The number of pregnancies in rats of widely different ages fed vitamin E deficient diets was found to be significantly lower than in controls raised on the same diet supplemented with alpha-tocopherol acetate (Kaunitz and Slanetz, '47). When single doses of alpha-tocopherol acetate were given to deficient, old females after mating, the number of pregnancies increased significantly (Kaunitz and Slanetz, '48). It was concluded that the failure of deficient females to become pregnant was due to disturbances of the implantation process rather than to failure of ovulation, fertilization or transport of the ova.

In order to test the validity of this conclusion and to obtain information on the ovarian function in vitamin E deficiency, it was decided to study the general reproductive phenomena in old, vitamin E deficient rats. The experiments were to include observations on the character of the female mating behavior, the number of ova ovulated and fertilized, and the rate of movement of the ova through the oviducts. Females of comparable ages from the same colony and maintained on the

[1] Aided by a grant from the Williams-Waterman fund of the Research Corporation.

same diet, but supplemented with alpha-tocopherol, served as controls.

MATERIALS AND METHODS

Twenty experimental and 22 control albino rats derived from a highly inbred colony ranging in age from 149 to 486 days were used. They represented the three to 5 generations maintained continuously on the semi-synthetic diet shown in table 1.

TABLE 1

Composition of vitamin E deficient diet

BASAL MIXTURE		SUPPLEMENTS TO BASAL MIXTURE	
	%		*mg/kg*
Casein, crude	30	Thiamine chloride	2
Cerelose	54	Riboflavin	4
Lard, commercial	10	Pyridoxine	4
Salt mixture [1]	4	Calcium pantothenate	10
Celluration	2	*p*-Aminobenzoic acid	300
		Choline	1000
		Inositol	1000
		Vitamin K	4
		Oleum percomorphum	200

[1] Hawk, Oser and Summerson ('47).

Since lard was the only measurable source of tocopherol in the diet it was estimated that the daily intake of tocopherol ranged between 20 to 40 μg.[2] This diet had previously proved satisfactory in studies of growth and bioassays (Kaunitz, '46; Kaunitz and Beaver, '46). The control animals were kept on the same diet supplemented by 3 mg of synthetic dl-alpha-tocopherol acetate [3] per 100 gm diet, allowing a daily intake of about 300 μg.

When the region of the iliac crest of a female rat in heat is manually stroked, or when she is mounted by a vigorous

[2] We are indebted to Dr. Philip L. Harris and Dr. Mary L. Quaife of Distillation Products, Inc., Rochester, New York, for their help in the assay of lard samples.

[3] We are indebted to Dr. Leo A. Pirk of Hoffman-La Roche, Inc., for supplying alpha-tocopherol and the other synthetic vitamins used.

male, the characteristic posture of lordosis is assumed. This posture is characterized by extension of the limbs, arching of the back and raising of the pudendal area and tail. The onset of heat can be accurately determined by this response. The relationship between the onset of heat and ovulation is sufficiently definite that the latter may be said to occur approximately 10 hours after the beginning of heat (Blandau, Boling and Young, '41; Boling, Blandau, Soderwall and Young, '41).

Females in heat were placed with vigorous males in order to observe the character of the heat response and to determine the time of insemination. At varying intervals after mating the females were killed and their reproductive tracts examined to ascertain the position of the ova, the total number ovulated and fertilized, and whether the fertilized ova were developing in a normal manner.

EXPERIMENTS

Observations on vitamin E deficient rats

The general character of the pro-estrous and estrous behavior of these females was similar to that of the controls. The onset of behavioral estrus in the older animals of both the experimental and control groups was neither as abrupt nor as intense as in younger females. In the majority of animals a definite lordosis response could be elicited either by manual manipulation or as the result of mountings of vigorous males (table 2). Four of the animals in which the behavioral response was not as vigorous eventually mated and fertilized ova were found later in their reproductive tracts. One of the females showed a typical male-like behavior characterized by frequent mounting and considerable genital exploration of other females and males. When this female was subsequently killed ovulation had not occurred; the ovaries, however, contained many large, possibly cystic follicles. One of the 20 females in this group died during the period of observation. Autopsy revealed that both oviducts and cornua contained large amounts of purulent material.

TABLE 2

Mating behavior, number of ova ovulated and fertilized, and their position in the reproductive tract of old vitamin E deficient rats

ANIMAL NUMBER	AGE	CHARACTER OF MATING BEHAVIOR	HOURS AFTER MATING ANIMAL KILLED	LOCATION OF OVA	TOTAL NO. OVA RECOVERED	NUMBER OF NORMAL OVA	NUMBER OF ABNORMAL OVA	REMARKS
	days							
RL–12	243	Vigorous	13	"Tubal sac"	6	6	0	all fertilized
L–25	260	Vigorous	15	"Tubal sac"	9	9	0	all fertilized
L–29	242	Weak	15	"Tubal sac"	7	6	1?	? fertilization one ovum
RL–4	149	Weak	24	Upper Isthmus	10	10	0	2 cell stage
RL–5	239	Vigorous	24	Upper Isthmus	9	9	0	2 cell stage
F–81	361	Vigorous	24	Upper Isthmus	9	8	1	8 normal ova
J–2	427	Vigorous	36	Upper Isthmus	7	7	0	2 cell stage
RL–3	243	Vigorous	36	Upper Isthmus	9	9	0	2 cell stage
L–14	350	Vigorous	48	Middle Isthmus	7	7	0	2 cell stage
FJ–31	399	Vigorous	48	Middle Isthmus	9	9	0	all fertilized
L–6	352	Vigorous	60	Distal Isthmus	8	6	2	2 fragmenting ova 5–4 cell; 1–3 cell
ST–2	352	Vigorous	96	Lower Isthmus	8	8	0	morula stage
F–12	306	Vigorous	96	Cornua	10	10	0	blastocysts
L–26	244	Vigorous	96	Cornua	8	5	3	morula stage 3 fragmenting
F–39	374	Weak	120	Cornua	3	0	3	3 fragmenting ova
J–10	404	Vigorous	120	Cornua	5	4	1	4 blastocysts 1 fragmenting
L–21	262	Weak	120	Cornua	7	5	2	5 blastocysts 2 fragmenting ova
L–13	381	Vigorous	192	....	0	0	0	both cornua filled with bloody fluid
Total					131	118	13	
average					7.2	6.5	0.7	
range					0–10	0–10	0–3	

A total of 131 ova were recovered from 18 females in this group (table 2). The ova generally were located within the segments of the reproductive tract expected for the particular time interval between ovulation and death (Huber, '15). For example, the ova of all animals examined up to 20 hours after ovulation were confined to the dilated loops of the ampulla and were still surrounded by a varying number of layers of granulosa cells. Ova of animals examined 48 or 72 hours after mating were located in the proximal and distal loops of the isthmus, respectively.

The rate of development in 118 (90%) of the 131 ova recovered appeared to be within normal limits. The remaining ova (13) were in various stages of fragmentation. Whether their death was due to failure of fertilization or to abnormal development after fertilization could not be determined.

Total	131	118	13	
average	7.2	6.5	0.7	
range	0–10	0–10	0–3	

Observations on control rats

Table 3 summarizes the findings with respect to the control group, which had been permitted a daily intake of about 300 μg alpha-tocopherol acetate. Three of the 22 females in this group died of unknown causes during the observation period. Two additional females failed to come into heat but subsequent examination of their ovaries showed several sets of recent corpora lutea. The general pattern of mating behavior was similar to that of the E deficient group. Two of the three females whose lordosis response was not as vigorous as that of younger, normal females nevertheless mated, and fertilized ova were recovered from their reproductive tracts. Another female, found to be in heat by manual manipulation, refused to mate with a vigorous male. Subsequent examination revealed that ovulation had occurred.

One hundred and sixteen ova were recovered from 17 females, 81%of which were developing normally. Six per cent of the ova recovered were not fertilized. The rate of transport of ova through the oviducts was normal.

TABLE 3

Mating behavior, number of ova ovulated, and their position in the reproductive tract of old rats raised on a semi-synthetic, "complete" diet

ANIMAL NUMBER	AGE	CHARACTER OF MATING BEHAVIOR	HOURS AFTER MATING ANIMAL KILLED	LOCATION OF OVA	TOTAL NO. OVA RECOVERED	NUMBER OF NORMAL OVA	NUMBER OF ABNORMAL OVA	REMARKS
	days							
H–87	393	Vigorous	15	"Tubal sac"	5	5	0	
D–80	405	Vigorous	20	"Tubal sac"	8	7	1	1 unfertilized
M–88	272	Vigorous	48	Middle Isthmus	5	0	5	not fertilized
KD–57	174	Weak	48	Middle Isthmus	7	7	0	2 cell
KD–46	361	Vigorous	48	Middle Isthmus	8	8	0	7–2 cell; 1–4 cell
KM–22	281	Vigorous	48	Middle Isthmus	8	8	0	2 cell stage
KK–18	271	Vigorous	48	Middle Isthmus	9	9	0	2 cell stage
KM–11	407	Weak	48	Middle Isthmus	10	4	6	4–2 cell; not fertilized
DH–27	373	Vigorous	60	Cornua	6	6	0	blastocysts
KK–35	272	Weak	72	Lower Isthmus	6	0	6	unfertilized
H–90	402	Vigorous	72	Lower Isthmus	8	7	1	7–2 cell; 1 not fertilized
D–82	403	Vigorous	96	Cornua	3	3	0	Normal implantation
KM–63	400	Vigorous	184	Cornua	8	8	0	Normal implantation
KK–45	278	Vigorous	184	Cornua	8	8	0	Normal implantation
K–23	486	Vigorous	192	Cornua	5	5	0	Normal implantation
K–19	377	Vigorous	192	Cornua	5	5	0	Normal implantation
DH–26	382	Vigorous	288	Cornua	7	4	3	3 empty implantation cavities
Total					116	94	22	
average					6.8	5.5	1.1	
range					3–10	0–9	0–6	

DISCUSSION

One of the most striking aspects of vitamin E deficiency in the rat is the testicular degeneration resulting in cessation of sperm production (Mason, '33). No comparable effect on the ovaries of rats has been observed (Stähler, Hebestreit and Fladung, '40). Ovulation and fertilization as well as the development of ova during their sojourn in the oviduct were shown in the present experiments to be normal even in old, deficient animals.

Wiesner and Bacharach ('37) reported that prolonged vitamin E deficiency reduces the intensity of reproductive behavior in male rats reared on an E deficient diet from the time of weaning. Similarly, Martin and Moore ('39) found that female rats deprived of vitamin E for prolonged periods showed abnormal estrous cycles. Although the length of the reproductive cycles was not determined in either group of animals in the present investigation, one obtained the impression that the cycles in both groups were considerably longer than in younger animals. However, when a female came into heat her behavioral response was of sufficient vigor to allow successful mating and insemination. The general decrease in the intensity of the behavioral response in both groups of animals is interpreted as an effect of aging, since a similar diminution of sexual behavior is commonly seen in older breeding animals.

The reduced ability of the vitamin E deficient rat to become pregnant appears to be caused by uterine rather than ovarian dysfunction. Intense pigmentation of the uterine smooth muscle is characteristic of advancing age in E deficient animals, yet results to date indicate that this does not seriously impair the physiological response of this organ to sympatho- and parasypathomimetic drugs. The possibility nevertheless exists that metabolic changes related to this phenomenon could impair the mechanism of implantation or early placental development (Martin and Moore, '39).

SUMMARY

In older vitamin E deficient female rats the pattern of mating behavior, number of ova ovulated and fertilized, and early development and transport of ova through the oviducts is comparable to that of females raised on the same diet supplemented by alpha-tocopherol. The low fecundity rate of vitamin E deficient rats is, therefore, apparently due to disturbances of the uterine implantation of the ovum rather than to failure of ovulation, fertilization, or transport of ova through the oviducts.

LITERATURE CITED

BLANDAU, R. J., J. L. BOLING AND W. C. YOUNG 1941 The length of heat in the albino rat as determined by the copulatory response. Anat. Rec., *79:* 453–463.

BOLING, J. L., R. J. BLANDAU, A. L. SODERWALL AND W. C. YOUNG 1941 Growth of the graafian follicle and the time of ovulation in the albino rat. Ibid., *79:* 313–332.

HAWK, P. B., B. OSER AND H. SUMMERSON 1947 Practical Physiological Chemistry. The Blakiston Co., Philadelphia, 12th ed. See p. 1273.

HUBER, G. C. 1915 The development of the albino rat, Mus norvegicus albinus. J. Morph., *26:* 247–358.

KAUNITZ, H. 1946 Influence of a single dose of alpha tocopherol administered to vitamin E deficient rats on the fifteenth day upon subsequent growth. J. Nutrition, *32:* 327–336.

KAUNITZ, H., AND J. J. BEAVER 1946 Tocopherol content of skeletal muscle: Comparison of chemical and bioassay methods. J. Biol. Chem., *166:* 205–217.

KAUNITZ, H., AND C. A. SLANETZ 1947 Influence of alpha tocopherol on implantation in old rats. Proc. Soc. Exp. Biol. Med., *66:* 334–337.

———— 1948 Implantation in normal and vitamin E deficient rats. J. Nutrition, *36:* 331–338.

MARTIN, A. P., AND T. MOORE 1939 Some effects of prolonged vitamin E deficiency in the rat. J. Hygiene, *39:* 643–650.

MASON, K. E. 1933 Differences in testis injury and repair after vitamin A deficiency, vitamin E deficiency, and inanition. Am. J. Anat., *52:* 153–240.

STÄHLER, F., E. HEBESTREIT AND K. FLADUNG 1940 Experimentelle und klinische Untersuchungen über das Vitamin E. Arch. Gyn., *170:* 142–172.

WIESNER, B. P., AND A. L. BACHARACH 1937 Effect upon sex behavior of a diet deficient in vitamin E. Nature, *140:* 972–973.